

**Alternatives to fish oil substitution - An assessment of strategies for
sustaining n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA)
levels in salmonids**

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**Submitted in fulfilment of
the requirements of the degree of
Doctor of Philosophy
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DECLARATION

This thesis contains no material which has been accepted for a degree or diploma by any tertiary institution. To the best of my knowledge the thesis does not contain any material written or published by another person, except where due reference is made.

Mohamed Basseer Codabaccus

AUTHORITY OF ACCESS

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ABSTRACT

The use of alternate oils (AO) in aquafeeds is now a reality due to the rise in the price of fish oil (FO). The lack of n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid (n-3 LC-PUFA) in AO is a major constraint due to the resulting low levels of n-3 LC-PUFA obtained in farmed fish. The aim of this study was to understand the metabolic basis underlying current and innovative strategies to maintain n-3 LC-PUFA levels in salmonids, particularly for Atlantic salmon, fed diets in which fish oil (FO) is substituted with AO in a series of four independent experiments. Use of *Echium* oil (EO) rich in stearidonic acid (SDA) has the potential to bypass the initial $\Delta 6$ desaturase enzyme which is a rate limiting step for n-3 LC-PUFA biosynthesis. This hypothesis was tested by growing Atlantic salmon in freshwater and seawater on diets where FO was completely substituted by either EO or rapeseed oil (RO) with comparison to a FO diet. The results indicated that SDA rich oil is a more suitable candidate for FO replacement in aquafeeds for Atlantic salmon compared to conventional vegetable oils due to enhanced n-3 LC-PUFA biosynthesis. The use of a FO finishing diet (FOFD) is a suitable way to restore n-3 LC-PUFA in fish after a growth period with AO. Preferential FA metabolism may contribute to n-3 LC-PUFA restoration by favouring β -oxidation of saturated fatty acids and/or monosaturated fatty acids. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) restoration was investigated in rainbow trout fed a diet where FO was substituted by 50% palm fatty acid distillate (PFAD) and 75% PFAD followed by a FOFD period. There was no evidence for preferential FA metabolism occurring and the dilution model was a good predictor of FA changes after dietary change. In aiming at improving the n-3 LC-PUFA restoration by the FOFD strategy, a short term food deprivation after growth on 75% PFAD prior to feeding the FOFD was undertaken for Atlantic salmon smolts. Short term food deprivation reduced lipid content especially in the fillet of fish which lead to an increase in n-3 LC-PUFA % composition.

Subsequent feeding to satiation with a FOFD improved the restoration of n-3 LC-PUFA in the fillet of unfed fish. This thesis also examined the use of oil blends in aquafeeds with emphasis on varied DHA and EPA ratio as a strategy for sustaining n-3 LC-PUFA in Atlantic salmon. The DHA and EPA ratio of FO is typically 1:1.5 in any blend of FO and AO. According to EPA and DHA metabolism in fish, higher dietary DHA content to EPA might be more suitable for optimizing their deposition. We tested whether altering the dietary DHA: EPA ratio in Atlantic salmon by increasing dietary DHA inclusion in conjunction with low dietary n-3 LC-PUFA content may enable efficient n-3 LC-PUFA deposition in fish. The results indicated that a diet having a higher DHA: EPA ratio than usually encountered in FO or FO/AO blend diets is better suited for optimizing n-3 LC-PUFA deposition in Atlantic salmon.

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LIST OF ABBREVIATIONS

The following abbreviations are used in this thesis:

AD, apparent digestibility
ALA, α -linolenic acid
ANOVA, analysis of variance
AO, alternate oils
ARA, arachidonic acid
CF, chicken fat
CMC, carboxymethyl cellulose
DHA, docosahexaenoic acid
DM, dry matter
DNA, deoxyribose nucleic acid
DPA, docosapentaenoic acid
EDTA, ethylenediaminetetraacetic acid
EFA, essential fatty acids
EO, *Echium* oil
ETA, eicosatetraenoic acid
FA, fatty acids
FAMB, fatty acid mass balance
FAME, fatty acids methyl esters
FC, total feed consumption
FER, feed efficiency ratio
FFA, free fatty acids
FID, flame ionisation detection
FL, fork length
FM, fishmeal
FO, fish oil
FOFD, fish oil finishing diet
FSI, fish in
FSO, fish out
GC, gas chromatography
GC-MS, gas chromatography mass spectrometry
GLA, γ -linolenic acid

GM, genetically modified
LA, linoleic acid
LC-PUFA, long-chain ($\geq C_{20}$) polyunsaturated fatty acids
MO, model oil
mRNA, messenger ribonucleic acid
MUFA, monounsaturated fatty acids
NMB, negative mass balance
OA, oleic acid
PFAD, palm fatty acid distillate
PL, polar lipids
PMB, positive mass balance
qRT-PCR, quantitative real time-polymerase chain reaction
RNA, ribose nucleic acid
RO, rapeseed oil
SDA, stearidonic acid
SEM, standard error of mean
SFA, saturated fatty acids
SGR, specific growth rate
TAG, triacylglycerols
TLC, thin layer chromatography
TLE, total lipid extract
UF, unfed
VO, vegetable oils
WG, weight gain
WW, wet weight

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

1.1 Introduction

Global capture fisheries have reached maximum sustainable yield and consequently, the catch has levelled off at around 90 million tonnes per annum (FAO, 2009). The high demand for fish from the ever increasing world population has thus propelled the aquaculture industry as the fastest growing animal food producing sector, with aquaculture set to overtake capture fisheries as the primary source of seafood (FAO, 2009). Research in fish nutrition has helped to establish optimum macro nutrient requirements for major carnivorous farmed fish species, such as salmonids (Halver and Hardy, 2002). The ability of salmonids to utilise high dietary levels of lipids (typically up to 40%), made possible by extrusion and vacuum coating technologies, has allowed a low optimum protein: energy ratio to be achieved. Until the past few years fish oil (FO) has been the main source of lipid in aquafeeds for carnivorous fish species especially from marine origin. Aquafeeds use 88% of total FO production, of which 56% is used in feed for salmonids, particularly for Atlantic salmon and rainbow trout (Tacon and Metian, 2008). The high demand for FO, mainly from the aquafeed industry, and the limited FO supply from wild caught fisheries has caused a sharp rise and large fluctuations in the price of FO during recent years (Naylor et al., 2009, Turchini et al., 2009, 2010). Besides the limited supply of FO, there are other concerns; the catch of wild fish is unpredictable due to the frequent occurrence of El Niño events and, depending on the FO sources, the presence of dioxins and PCBs (Naylor et al., 2009). Therefore, to sustain the aquaculture industry, FO substitution in aquafeeds for major farmed carnivorous fish species, most particularly for salmonids, is nowadays a reality (Naylor et al., 2009; Turchini et al., 2009, 2010). The aim of this chapter is to highlight the key issues relating to FO substitution in aquafeeds with emphasis on salmonids.

1.2 *Salmonids*

As salmonids are the principal consumer of FO, substitution of dietary FO for salmonids has become an industry priority (Gatlin et al., 2007; Miller et al., 2008a; Turchini et al., 2009). This thesis has a principal focus on Atlantic salmon which is the main cultured salmonid species. Being a carnivorous fish species, the main ingredients in Atlantic salmon feeds have been fishmeal (FM) and FO. Consequently, the fish in/fish out (FSI/FSO) ratio is relatively high (3.2-5.0) for farmed Atlantic salmon (Black, 2001; Naylor et al., 2000, 2009) and has thus raised concerns on the ecological impact of such practice to the environment. However, aquaculture practice has an ecological advantage over wild caught Atlantic salmon since 1 kg of growth in the wild is equivalent to 10-15 kg of fish eaten as prey or captured as by-catch (Miller et al., 2008a). FM substitution in aquafeeds is also an industry priority, but that of FO substitution is probably most pressing and more likely to have an impact on reducing the FSI/FSO ratio. Reducing FO by 4% in salmon feeds leads to a drop of FSI/FSO from 5.0 to 3.5; in contrast, a 4% reduction of FM in diets only moderately reduced FSI/FSO from 5.0 to 4.8 (Naylor et al., 2009). Therefore, the amount of forage fish used to produce feed for salmon is driven by the need for FO to a far greater extent than FM (Naylor et al., 2009).

Atlantic salmon is an anadromous fish and spends its early life cycle in freshwater. In freshwater, the natural diet for salmonids is composed of fatty acids (FA) from limnic origin which is rich in n-6 and n-3 short-chain ($\leq C_{18}$) PUFA, while in seawater the dominant dietary FA are the n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA). This environmental difference in the natural dietary FA composition may have profound effects on the regulation of key enzymes involved in

the FA metabolism of Atlantic salmon. An understanding of the environmental and nutritional regulation of key FA metabolic enzymes is therefore critical when substituting FO in Atlantic salmon. All fish including salmonids are ectotherms and as such, temperature largely affects their physiology including FA metabolism. Since increasing water temperatures are frequently encountered as a result of climate change, the effect of temperature rise on FA metabolism particularly that of n-3 LC-PUFA deposition is of growing interest (Miller et al., 2008a).

1.3 Alternate oils – the challenge

Suitable alternate oils (AO) for FO should satisfy four main criteria and be:

- less expensive
- sustainable
- free of contaminants
- accepted by consumers as ingredients in aquafeeds.

Vegetable oils (VO) satisfy these main criteria and, as such, are the prominent substitute for FO in aquafeeds. The use of fat from rendered animals represents another alternative, but current regulations in European countries/market forbid its use. In non European Union countries, including Australia, legislation is less severe and allows the use of rendered products (Miller et al., 2008a). The common consensus is that, while there is a pressing need to substitute FO in aquafeeds, AO should not compromise the growth and health of the fish nor the health promoting benefits of consuming fish (Gatlin et al., 2007; Miller et al., 2008a). Extensive research has been conducted on AO in salmonids diets and AO rarely affected fish growth or fish health as long as their (n-3 LC-PUFA) requirements are met (Rosenlund et al., 2001; Bransden et al., 2003; Torstensen et al., 2005). The main

concern, however, is the decrease of n-3 LC-PUFA content in fish, more particularly eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), with increasing levels of FO substitution, which in turn may reduce the health promoting benefits for humans when consuming fish (Seierstad et al., 2005). The rationale for such observations is that AO, unlike FO, are devoid of n-3 LC-PUFA and since the FA profile of fish is mostly the reflection of its dietary FA profile, complete substitution by FO in aquafeeds is presently not desirable. FO is obtained from wild fish, but fish do not synthesise n-3 LC-PUFA themselves, rather LC-PUFA are bioaccumulated from the food chain with the original source being at the lowest trophic level of the food chain – microalgae and thraustochytrids, and selected bacteria. During the past few years, research has focused on understanding the lipid metabolism of fish, particularly for salmonids, in order to develop strategies for maintaining high levels of n-3 LC-PUFA in fish fed AO (Tocher, 2003a).

1.4 Strategies to maintain high n-3 LC-PUFA levels

1.4.1 Endogenous n-3 LC-PUFA biosynthesis

All vertebrates including fish are able to biosynthesise n-3 LC-PUFA from their precursors FA using a series of desaturase and elongase enzymes along the n-3 pathway. α -linolenic acid (ALA; 18:3n-3) is the first FA in the n-3 pathway and is an essential fatty acid (EFA), therefore has to be provided by the diet. Some VO such as rapeseed, linseed and soybean oils possess ALA in sufficient amounts for endogenous n-3 LC-PUFA biosynthesis to occur. Previous studies on Atlantic salmon have demonstrated that when sufficient dietary ALA is supplied using VO, there is an increase in hepatic ALA bioconversion to EPA and DHA compared to fish fed on FO diet; bioconversion coincides with the smoltification period and peaks around seawater transfer (Bell et al., 1997; Tocher et al., 2000; 2003b). After seawater

transfer, the biosynthetic activity decreases and may suggest possible environmental regulation of n-3 LC-PUFA. The difference in biosynthetic activity is an evolutionary consequence of the availability of n-3 LC-PUFA rich diets in seawater (Sargent et al., 2002). In Atlantic salmon three quarter of the production cycle occurs in seawater, therefore when FO is substituted by VO, n-3 LC-PUFA levels decrease markedly in seawater fish. The conversion of ALA to stearidonic acid (SDA; 18:4n-3) by the $\Delta 6$ desaturase enzyme is the first step along the n-3 pathway and is a rate limiting step (Brenner, 1981). Theoretically if enough dietary SDA is present, this first rate limiting step can be by-passed resulting in more efficient n-3 LC-PUFA biosynthesis. This hypothesis has been verified in Atlantic salmon parr in freshwater fed *Echium* oil (EO), which is naturally rich in SDA (Miller et al., 2007). The initial result was promising since there was no difference in EPA and DHA content in the muscle between fish fed EO diet or FO diet. In that study, FO was completely substituted by EO and FM was defatted to reduce dietary n-3 LC-PUFA to negligible amounts. The reduction of dietary n-3 LC-PUFA content is regarded as key when investigating the endogenous n-3 LC-PUFA biosynthetic capacity of fish since dietary n-3 LC-PUFA may lead to inhibition of n-3 LC-PUFA biosynthesis. As mentioned earlier, the ability for freshwater fish and particularly Atlantic salmon undergoing smoltification to biosynthesize n-3 LC-PUFA is higher compared to when they are in seawater. Therefore, in a follow-up experiment, Atlantic salmon smolt fed EO diet in seawater had lower EPA and DHA content in whole carcass and muscle compared to fish fed FO (Miller et al., 2008b). However, liver EPA content was not different between fish fed on either EO or FO based diets and up-regulation of $\Delta 5$ desaturase and elongase gene expression also occurred. Thus, the increased n-3 LC-PUFA biosynthesis in the liver was not enough to increase its deposition in the whole carcass and muscle (Miller et al., 2008b). These two studies were independent; the missing scenario is an

experiment that encompasses growing Atlantic salmon from parr through to smolt from freshwater to seawater. Consequently this was addressed in the current thesis (Chapters 2 and 3). In Chapter 2, the use of a recently developed fatty acid mass balance approach (Turchini et al., 2007) was used to verify the hypothesis that increased n-3 LC-PUFA biosynthesis occurs in SDA fed fish at the whole body level and that environment can effect n-3 LC-PUFA biosynthesis. In Chapter 3, the measurement of gene expression of n-3 LC-PUFA biosynthetic enzymes in the liver and muscle was used as a tool to examine the underlying mechanism of n-3 LC-PUFA biosynthesis in SDA fed fish with possible environmental influences on n-3 LC-PUFA biosynthesis also investigated.

1.4.2 Blended oil

The use of an oil blend comprising FO and AO in aquafeeds is probably the most popular strategy to maintain high levels of n-3 LC-PUFA in salmonids as it is suited for a broad range of AO (Miller et al., 2008a; Turchini et al., 2009). This strategy is principally based on two important observations from our knowledge of fish lipid metabolism: 1) Saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) are preferred substrates for β -oxidation (Turchini et al., 2009); 2) FA β -oxidation preference is subservient to surplus dietary FA (Stubhaug et al., 2007; Chapter 2 and Codabaccus et al., 2011). In Atlantic salmon, both EPA and DHA is extensively used for β -oxidation when in surplus (Stubhaug et al., 2007; Chapter 2 and Codabaccus et al., 2011). Feeding Atlantic salmon exclusively on FO can thus be regarded as a wasteful practice due to the high β -oxidation of n-3 LC-PUFA. Blending AO with FO favours β -oxidation of preferred substrates (SFA and MUFA) and simultaneously prevents dietary n-3 LC-PUFA levels to be present in surplus. Judicious choice and inclusion levels of oils in blends are key issues that have to be

addressed when using this strategy. Palm based oils and animal fats are rich in SFA, while other VO have high levels of MUFA and/or short-chain ($\leq C_{18}$) PUFA (rapeseed, linseed, soybean and sunflower oils). Presently there are no clear standards on the inclusion levels of AO for aquafeeds. Previous studies have shown that an inclusion level in excess of 25-50% from various VO sources in diets causes a reduction in n-3 LC-PUFA in Atlantic salmon as compared to fish fed on FO (Bell et al., 2001, 2002, B ransden et al., 2003, Menoyo et al., 2005, 2007). Therefore it is very difficult to designate a threshold on the level of substitution because it depends primarily on the AO used and also to what extent a reduction in n-3 LC-PUFA is considered detrimental. A reduction in n-3 LC-PUFA levels is not an issue for the fish in terms of health or growth as long as dietary n-3 LC-PUFA requirements are met, but rather raises concern from a human health benefit aspect. The level of FO substitution by AO seems to be dictated by the balance between the economic considerations and also FO availability versus the health benefits of consuming fish. A common point of reference remains the n-3 LC-PUFA levels in wild fish compared to farmed fish. S ince farmed fish generally contains more fat than their wild counterparts due to the high energy rich diets used, even if a significant amount of FO is replaced by VO, farmed fish will still have high n-3 LC-PUFA content.

While n-3 LC-PUFA content in fish is a major issue with FO substitution in aquafeeds, fish growth is probably as, if not more important. This raises the question of digestibility of FA classes in relation to availability of energy. The apparent digestibility (AD) of SFA is generally $< \text{MUFA} < \text{PUFA}$ and is further exacerbated at low water temperatures. In this context, palm based oils; rich in SFA, at high inclusion levels might not be suited as AO for cold water species such as salmonids (Ng et al., 2003; 2004; 2007). However, with likely climate change effects, elevated

water temperatures (e.g. 19°C) are now often encountered worldwide including in Tasmanian waters (Miller et al., 2006; 2008a), therefore the impact of climate change may positively contribute to enhance energy availability from SFA.

The use of most VO (rapeseed, linseed, soybean and sunflower oils) in blends for salmonids is appropriate with regards to energy availability from FA since they contain predominantly MUFA and short-chain PUFA to varying degrees. There is an increasing awareness on the positive health benefits of a high n-3: n-6 ratio in human diets (Simopoulos, 2002; Goodstine et al., 2003). The presence of high levels of n-6 PUFA present in most VO, mostly as linoleic acid (LA; 18:2n-6) results in a decrease in the n-3/n-6 ratio in fish fed on these VO diets rich in LA is yet another constraint.

The blended oil strategy was investigated in Chapter 6 together with the introduction of a new concept “n-3 LC-PUFA saving” aiming at further optimizing n-3 LC-PUFA deposition.

1.4.3 Fish oil finishing diet

Growing fish on diets comprising a blend of VO and FO for most of the production cycle and then finishing off with a FO based diet for a period prior to harvest is another strategy to restore n-3 LC-PUFA levels. As opposed to the blended oil approach, in a FO finishing diet (FOFD) strategy, high levels of FO substitution during the grow-out period may be used (Pratoomyot et al., 2008), typically $\geq 75\%$ of total oil, provided n-3 LC-PUFA requirements are met. Since the fish FA profile is the reflection of dietary FA profile, after the grow-out period, fish n-3 LC-PUFA levels will be low and FA from AO will predominate. The effectiveness of n-3 LC-

PUFA restoration during the FOFD period will then depend primarily on the duration of the FOFD period and nutritional history of the grow-out diet. Efficient restoration of EPA and DHA in Atlantic salmon fillet using FOFD has been well documented (Bell et al., 2003a, b; 2004; Torstensen et al., 2004, 2005), but generally complete EPA and DHA restoration was not achieved when compared to fish fed a FO diet throughout. The FA change after dietary change represents the dilution of existing FA stores (from grow-out diet), and the tissue FA composition of fish will resemble that of the diet after a certain feeding period (Robin et al., 2003; Jobling et al., 2004a). Therefore if the dilution theory prevails, the restoration of n-3 LC-PUFA levels is entirely depended on the dietary inclusion level of the alternate oil during the grow-out period and the duration of the finishing FO period. To this end, a dilution model has been proposed whereby the fillet FA composition after dietary change can be predicted (Robin et al., 2003). However, a deviation to the dilution model has been observed (Jobling, 2004b; Lane et al., 2006; Turchini et al., 2006), suggesting that preferential FA metabolism is a contributing factor to FA changes occurring after dietary change. Preferential FA metabolism is regarded as key as it may accelerate EPA and DHA restoration by preferentially utilising existing FA stores (from grow-out diet) as energy source. Oils composed of primarily SFA and MUFA are regarded as better suited as AO in diets during the grow-out period prior to applying the FOFD (Turchini et al., 2009). In this context, palm-based oils or rendered animal fats may be the best AO candidates, albeit the low apparent digestibility (AD) of SFA at low water temperature. In the advent of global climate change, higher water temperatures are encountered and this may also contribute towards preferential FA metabolism. In addition, palm fatty acid distillate (PFAD), which is a by-product of the refining of crude palm oil, contains ~80% free FA (Bahurmiz and Ng; 2007) and such a structure of the constituent FA has the potential to improve the AD of SFA (Ng et al., 2010).

The use the FOFD strategy at optimal and elevated water temperatures was investigated in Chapter 4 with PFAD as AO. In this study, rainbow trout was used as the test species since research using the FOFD strategy is limited for this salmonid species, a major aquaculture species that has a relatively high amount of FO in the diet (Chapter 4).

Hypothetically there exists another way to improve restoration of n-3 LC-PUFA when using the FOFD. A short term food deprivation to reduce the initial lipid content in fish after growth on the AO diet prior to commencing feeding on the FOFD may lead to higher n-3 LC-PUFA restoration (Palmeri et al., 2009). A short term food deprivation did not reduce the initial fillet lipid content in Murray cod; consequently this hypothesis could not be verified (Palmeri et al., 2009). In Atlantic salmon, fillet lipid has been shown to be readily used as an energy source upon starvation (Einen et al., 1998), therefore this hypothesis is best suited for testing with Atlantic salmon. As highlighted above with logistics issues working with harvest size Atlantic salmon, this strategy was examined on a conceptual basis with Atlantic salmon smolts (Chapter 5).

1.4.4 Genetically modified plants containing n-3 LC-PUFA rich oil

The different strategies mentioned so far may be considered as short to medium term solutions because complete substitution of FO in aquafeeds is not possible. As aquaculture is expected to grow further, in the long term a more sustainable source of n-3 LC-PUFA other than FO obtained from the limited wild fish stocks should be sought. Genetic engineering of oilseed crops to produce n-3 LC-PUFA by insertion of genes encoding for encoding desaturases and/or elongases have been suggested as a sustainable alternative source of n-3 LC-PUFA for use in aquafeeds (Miller et al., 2008a). This biotechnological approach is complex since it

involves the coordination of multiple gene expression encoding for different key enzymes of the n-3 biosynthetic pathway. Research during the first half of this decade resulted in low yields of n-3 LC-PUFA (Abbadi et al., 2001; Robert et al., 2005; Sayanova & Napier, 2004). Recently, more progress has been achieved and the yield of n-3 LC-PUFA has reached 20 % EPA and 3.3% DHA from soybean (Damude & Kinney, 2007) and 25% EPA and 13 % DHA from oilseed crops or model plants (Nichols et al., 2010).

In Chapter 6, a new concept of n-3 LC-PUFA “saving” is introduced. It is very common to observe a change in dietary ratio of DHA: EPA compared to fish DHA: EPA ratio, from being low in diets (< 1) to high in fish (> 1) (Bransden et al., 2003; Miller et al., 2008b; Chapter 2 and Codabaccus et al., 2011). This feature is principally as a result of surplus dietary EPA β -oxidation as opposed to the rather conservative nature of DHA in fish. Subsequently, a diet comprising a higher DHA: EPA ratio (> 1) may promote more efficient deposition of n-3 LC-PUFA in fish. Research in this area is limited, most probably due to its practical significance since no such oils with this desired FA characteristic is presently commercially available. However, in a GM model crop, it was shown that DHA may be higher than EPA (Petrie et al., 2010). Therefore it is of interest to investigate the effect of high dietary DHA: EPA ratio on n-3 LC-PUFA deposition in fish (Chapter 6). Fish culture experiments aiming at “saving” n-3 LC-PUFA such as that in Chapter 6 would be very informative to scientist in the field of plant genomics as it might be possible to tailor-made oils in aquafeeds not only for Atlantic salmon but to major cultured fish species. In the near future, genetically modified (GM) plants may provide the most economically viable and sustainable source of n-3 LC-PUFA in aquafeeds (Miller et al., 2008a). While significant progress has been achieved in this field, in order to use

oils from GM plants, several key issues ranging from government policies, and industry and consumer acceptance will need to be addressed. However, it is vital to conduct preliminary research by blending available oils to mimic the FA composition of future GM oils. In Chapters 6 the concept of n-3 LC-PUFA “saving” was investigated by using blends of tuna oils (rich in DHA), rapeseed oil and mackerel oil (typical FO) to obtain high dietary DHA: EPA ratio.

1.5 Aim and objectives of thesis

The overall aim of this study was to understand the metabolic basis underlying current and innovative strategies to maintain n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid (LC-PUFA) levels in salmonids fed diets in which fish oil (FO) is substituted with stearidonic acid (SDA) rich oil (Chapters 2 and 3), palm fatty acid distillate (PFAD) (Chapters 4 and 5), blend of tuna oil, rapeseed oil and mackerel oil.

To address this aim, the following strategies were investigated:

- 1) The use of dietary stearidonic acid as a precursor for n-3 LC-PUFA biosynthesis in Atlantic salmon grown from parr to smolt (Chapters 2 and 3)
- 2) The use of a fish oil finishing diet strategy in rainbow trout previously fed on different dietary levels of palm fatty acid distillate at optimal and elevated water temperatures (Chapter 4)
- 3) Use of a short term food deprivation in Atlantic salmon smolt after grow-out on palm fatty acid-based diet prior to feeding on fish oil finishing diet improve the efficiency of EPA and DHA restoration (Chapter 5)

- 4) The use of high dietary DHA: EPA ratio by using blends of blend of tuna oil, rapeseed oil and mackerel oil for efficient deposition n-3 LC-PUFA (Chapter 6)

The strategies listed above were based on specific hypotheses that were investigated in four separate nutrition experiments. Chapters 2-6 concern these experiments which are written as stand alone chapters that have been published or are in preparation for submission to international journals.

Chapter 2 relates to the use of dietary SDA as a biosynthetic precursor to n-3 LC-PUFA at the whole organism level of Atlantic salmon. This experiment encompassed growing Atlantic salmon on high dietary SDA from parr to smolt in freshwater and seawater; hence, it is the most complete work on the effect of dietary SDA on lipid metabolism for Atlantic salmon. This study has been accepted for publication in the British Journal of Nutrition and is in press. Chapter 3 investigated the use of dietary SDA as a biosynthetic precursor to n-3 LC-PUFA at a tissue and organ level (muscle and liver) for Atlantic salmon. Samples for this study came from the same feeding experiment as in chapter 2. The gene expression of key enzymes involved in the n-3 LC-PUFA biosynthetic pathway was also measured to examine the nutritional and environmental regulation of these genes especially with respect to dietary SDA.

Chapter 4 investigates the effectiveness of a FOFD strategy to restore EPA and DHA in rainbow trout previously fed on different levels of PFAD. The effect of temperature on the restoration process was studied as well as assessing whether a dilution model was a good predictor of FA change following dietary change.

Chapter 5 represents a modification of the FOFD strategy which verified whether a period of short term food deprivation prior to starting the FOFD would improve the efficiency of EPA and DHA restoration in Atlantic salmon smolts. The hypothesis here is that food deprivation will lead to β -oxidation of existing FA stores from previous nutrition history, in this case a PFAD based diet; when starting with the FOFD, n-3 LC-PUFA deposition will then be more efficient.

Chapter 6 assesses the potential for future oils from genetically modified plants to replace FO in aquafeeds. Here, the deposition of n-3 LC-PUFA in Atlantic salmon in seawater was examined by using two model oils in diets which mimic the current trends in FA profile of genetically modified plant producing oils. In conjunction, the model oil treatments were also compared to fish fed on a blend of chicken fat and FO which is now a common practice in Tasmania.

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CHAPTER 2

Effect of feeding Atlantic salmon (*Salmo salar* L.) a diet enriched with stearidonic acid from parr to smolt on growth and n-3 long-chain ($\geq C_{20}$) PUFA biosynthesis.

Adapted from Codabaccus, M.B., Bridle, A.R., Nichols, P.D., Carter, C.G., 2011. Effect of feeding Atlantic salmon (*Salmo salar* L.) a diet enriched with stearidonic acid from parr to smolt on growth and n-3 LC-PUFA biosynthesis. Br. J. Nutr. 105, 1772-1782.

2.1 ABSTRACT

Vegetable oils (VO) have become the predominant substitute for fish oil (FO) in aquafeeds, however, the resultant lower content of n-3 long-chain ($\geq C_{20}$) PUFA (n-3 LC-PUFA) in fish has put their use under scrutiny. The need to investigate new oil sources exists. This research tested the hypothesis that in Atlantic salmon a high intake of stearidonic acid (SDA) from *Echium* oil (EO) would result in increased n-3 LC-PUFA biosynthesis due to lower requirement for $\Delta 6$ desaturase. Comparisons were made to fish fed on diets containing rapeseed oil (RO) and FO in freshwater for 112 d followed by 96 d in seawater. EO fish had higher whole carcass SDA and eicosatetraenoic acid (ETA) in freshwater and prolonged feeding on EO diet in seawater resulted in higher SDA, ETA, EPA and docosapentaenoic acid (DPA) compared to RO fish. Fatty acid mass balance of freshwater fish indicated higher biosynthesis of ETA and EPA in EO fish compared to fish on the other diets and a 2 fold increase of n-3 LC-PUFA synthesis compared to RO fish. In seawater, n-3 biosynthetic activity was low, with higher biosynthesis of ETA in EO fish and appearance of all desaturated and elongated products along the n-3 pathway. SDA enriched VO are more suitable substitutes than conventional VO from a human consumer perspective due to the resulting higher SDA content, higher total n-3 and improved n-3: n-6 ratio obtained in fish, although both VO were not as effective as FO in maintaining EPA and DHA content in Atlantic salmon.

2.2 INTRODUCTION

Aquaculture has expanded rapidly over the past decades with an average growth rate of 8.8 percent per year since 1970 compared with only 1.2 percent for capture fisheries (FAO, 2007). This increase in fish production has led to an increase in aquafeed production concurrent with a greater demand for fish oil (FO) and fishmeal (Naylor et al., 2000; Miller et al., 2008a). Consequently, the rise in FO demand from aquafeed industries has added further pressure on wild fisheries which are generally considered to be finite, fully exploited and at times unpredictable due to the El Niño events (Naylor et al., 2000, 2009). It is predicted that the future needs of the aquaculture industry for FO will outstrip current supply within the next decade (Pickova and Morkore, 2007). In addition to the predicted shortfall in FO supply, there has been concern about the levels of dioxins and dioxin like PCB's (polychlorinated bi-phenyls) in some FO depending on the source fishery (Jacobs et al., 2002; Bell et al., 2005) which presents a potential health hazard. Therefore the aquaculture industry is faced with a major challenge in finding suitable oil sources for replacement of FO (Miller et al., 2008a; Naylor et al., 2009).

In an effort to sustain Atlantic salmon aquaculture, a wide variety of commercial vegetable oils (VO) have been investigated as FO replacements (Miller et al., 2008a; Turchini et al., 2009). Use of VO rarely affects fish growth performance (Bell et al., 2002; Bransden et al., 2003; Torstensen et al., 2004). However, the low levels of n-3 long-chain ($\geq C_{20}$) PUFA (n-3 LC-PUFA), in particular EPA and DHA in fish fed on VO remains a major shortcoming. Generally with increasing increments of VO in diets, there has been a corresponding decrease of n-3 LC-PUFA content in fish. In Atlantic salmon fed 100% VO, flesh EPA and DHA levels were reduced to 30

and 35% respectively (Bell et al., 2003), because oils derived from vegetable sources lack n-3 LC-PUFA and the capacity for fish especially of marine origin to endogenously biosynthesize n-3 LC-PUFA from the VO substrates is limited (Miller et al., 2008b). In addition, VO are usually characterized by high levels of n-6 PUFA and low n-3: n-6 ratios, hence feeding diets high in VO has the potential to reduce the important health benefits derived from consumption of n-3 LC-PUFA obtained by eating fish and other seafood (Simopoulos, 2002; Ruxton et al., 2004).

So far there has been only one report of endogenous biosynthesis of n-3 LC-PUFA from metabolic precursors capable of matching levels of EPA and DHA present in fish fed a FO diet. The flesh of Atlantic salmon parr (in freshwater) fed *Echium* oil (EO) rich in stearidonic acid (SDA; 18:4n-3), a precursor of EPA, contained comparable levels of EPA and DHA to that obtained using a FO diet (Miller et al., 2007). However, in a follow-up trial with smolt, high levels of n-3 LC-PUFA usually found in seawater Atlantic salmon fed diets rich in FO were not attained via biosynthesis from precursors in the EO diet, though gene expression of $\Delta 5$ desaturase and elongase enzymes in the liver were upregulated (Miller et al., 2008b). Nutritional history might be important and the smolts had not been fed SDA rich diets prior to the EO diet (Miller et al., 2008b). In this study, we attempt to determine whether feeding EO from parr to smolt would result in increased n-3 LC-PUFA biosynthesis. A whole body fatty acid mass balance (FAMB) approach (Turchini et al., 2006, 2007, 2009) was used to investigate the metabolism of individual fatty acids along the n-3 pathway.

2.3 MATERIALS and METHODS

2.3.1 *Experimental diets*

Three diets were formulated to compare rapeseed oil (RO), *Echium* oil (EO) and fish oil (FO) (Table 2.1). Fish meal (Skretting Australia, Cambridge, Tasmania, Australia) was defatted using a mixture of hexane and ethanol (400 ml/100 ml fish meal). *Echium* oil was provided as Crossential SA14 (Croda Chemicals, East Yorkshire, UK). FO was from Jack mackerel, *Trachurus symmetricus* L., (Skretting Australia, Cambridge, Tasmania, Australia) and a domestic rapeseed oil was used (Steric Trading Pty Ltd, Villawood, NSW, Australia). The diets were manufactured using a California Pellet Mill (CL-2), dried and stored at -5°C (Carter et al., 2003b).

2.3.2 *Growth experiment*

The experiment was conducted at the University of Tasmania (Launceston, Tasmania, Australia) in accordance with University of Tasmania Animal Ethics guidelines (Investigation A0009731). Atlantic salmon (*Salmo salar* L.) parr (~25 g) were obtained from Wayatinah Salmon Hatchery (SALTAS, Tasmania, Australia) and acclimated for 14 days in a 3000 litres partial recirculation system. Fish were maintained on the FO diet (Table 2.1) prior to starting the experiment.

At the start of the experiment, fish were anaesthetized (50 mg/l, benzocaine), weighed and fork length measured. Eight fish were euthanized (100 mg/l) and stored at -20°C for measurement of initial fatty acid and chemical composition of whole carcass. The experiment used a partial recirculation system, equipped with a protein skimmer and physical, UV and biological filters (Bransden et al., 2003). Water temperature was kept constant at 15°C with continuous daily replacement of

approximately 15% volume. Dissolved oxygen, pH, ammonia, nitrate and nitrite were monitored daily to ensure water quality remained within parameters recommended for Atlantic salmon (Wedemeyer, 1996). Forty four fish were randomly allocated into each of twelve 300 litre tanks and the three diets were hand fed in quadruplicate at a fixed ration of 2.0% body weight per day (%BW/day) in two equal rations. Every 14 days, feed intake was monitored to adjust feeding ration. Since experimental fish had missed the natural window for smolting, photoperiod was manipulated following normal commercial procedures to trigger smoltification. At 28 days intervals, fish were bulk weighed and sixteen fish per treatment were weighed and fork length measured for calculation of condition factor (K). Based on physical characteristics of fish undergoing parr-smolt transformation such as silvering of the body, loss of parr marks and darkening of fin margins (McCormick et al., 2000), fish were transferred to seawater at 112 days. Prior to seawater transfer, fish were bulk weighed and two fish per tank were euthanized (100 mg/l benzocaine) and stored at -20°C for fatty acid and chemical composition analyses of whole carcass. At seawater transfer one FO replicate tank had less fish than expected (due to escapees). Data from this tank were omitted from analysis due to the different feeding history. After 7 day in seawater, fish were randomly culled to 24 fish/tank and blood from five fish per treatment was taken from below the anal fin with a heparinised syringe for measurement of plasma osmolality on a Vapro 5250 vapour pressure osmometer to confirm smolt status of fish. Blood plasma osmolality values for all groups (314-358 mOsm/kg) were within the range considered to be normal for Atlantic salmon smolts (Arnesen et al., 1998). At the end of the experiment (196 days), fish were bulk weighed and three fish per tank were euthanized (100 mg/l benzocaine) and stored at -20°C for whole carcass fatty acid and chemical composition analyses.

Specific growth rate (SGR) was calculated as $SGR (\%/d) = 100 * (\ln BW_f / \ln BW_i) / d$ where BW_f and BW_i are final and initial wet weights (g) and d the number of days of the experiment. Feed consumption (FC) was calculated as the total average amount of feed (g) consumed per fish over the number of days of the experiment. The feed efficiency ratio was calculated as $FER (g/g) = \text{total weight gain (g)} / FC (g)$. Condition factor (K) was calculated as $K (\%) = 100 * (BW / FL^3)$, where FL was the fork length (cm).

2.3.3 Digestibility

Diets included yttrium oxide (1 g/kg) as a digestibility marker (Carter et al., 2003a). On day 108-111 (freshwater phase), faecal samples from all tanks were collected from faecal settlement collectors (Guelph system) attached to the tanks between 1100-1700 and 1900-0900 h (Carter et al., 2003a, Ward et al., 2005). At the end of the experiment (seawater phase), fish were stripped for collection of faeces (Percival et al., 2001). Faecal samples were freeze-dried prior to chemical analysis. Apparent digestibility (AD) was calculated using the standard formula $AD (\%) = 100 - [100 (Y_{\text{diet}} / Y_{\text{faeces}}) * (FA_{\text{faeces}} / FA_{\text{diet}})]$ where Y is the % of yttrium oxide and FA is the % of particular fatty acids (Maynard & Loosli, 1969).

2.3.4 Chemical analysis

Standard methods were used to determine dry matter (DM) (freeze dry to constant weight followed by drying at 135°C for 2 h) (AOAC, 1995); total lipid (Bligh and Dyer, 1969); nitrogen (Kjedhal using selenium catalyst; crude protein was calculated as $N \times 6.25$); energy (bomb calorimeter, Gallenkamp Autobomb, calibrated with benzoic acid) and ash by combustion at 600°C for 2 h (AOAC, 1995). Apart

from DM, freeze dried samples were used for chemical analyses and corrected for DM.

Table 2.1: Ingredient and lipid composition (g/kg dry matter) of Atlantic salmon fed EO, FO and RO

	Diet		
	EO	FO	RO
<i>Ingredient composition (g /kg)</i>			
Fishmeal (defatted) ¹	250	250	250
Casein ²	50	50	50
Wheat gluten ³	100	100	100
Soybean meal ⁴	189	189	189
Fish oil ¹	0	200	0
Rapeseed oil ⁵	0	0	200
Echium oil ⁶	200	0	0
Pre gel starch ⁷	127	127	127
Vitamin mix ⁸	3	3	3
Mineral mix ⁹	5	5	5
Stay C ¹⁰	3	3	3
Choline chloride ¹¹	2	2	2
Sipernat ¹²	40	40	40
CMC ¹¹	10	10	10
Monobasic calcium phosphate ¹¹	20	20	20
Yttrium oxide ¹¹	1	1	1
<i>Chemical composition (g/kg DM)</i>			
Dry matter	911.3	905.0	907.7
Crude protein	342.3	346.8	345.8
Total lipid	213.0	215.9	213.7
Energy (MJ/kg DM)	19.7	19.7	19.7
<i>g/ kg DM</i>			
Total SFA	24.7	52.9	20.9
Total MUFA	36.8	51.1	123.5
18:3n-3 ALA	41.0	2.0	14.4
18:4n-3 SDA	15.7	4.3	0.1
20:5n-3 EPA	0.3	28.3	0.9
22:6n-3 DHA	0.4	9.8	0.6
Total n-3	57.4	50.3	16.1
18:2n-6 LA	36.2	9.4	41.4
18:3n-6 GLA	14.6	0.6	0.1
Total n-6	51.1	13.0	41.5
Total PUFA	108.5	67.5	57.7

¹Skretting Australia, Cambridge, Tasmania, Australia, ²MP Biomedicals Australasia Pty Ltd, Seven Hills NSW, Australia, ³Starch Australasia, Lane Cove, NSW, Australia, ⁴Hamlet Protein A/S, Horstens, Denmark, ⁵Croda Chemicals, East Yorkshire, UK, ⁶Steric Trading Pty Ltd, Villawood, NSW, Australia. ⁷Penford Limited, Lane Cove, NSW, Australia, ⁸Vitamin mix (ASV4) supplied per kilogram of feed: 2.81 mg thiamine HCl, 1.0 mg riboflavine, 9.15 mg pyridoxine HCl, 25 mg nicotinic acid, 54.32 mg calcium D-pantothenate, 750 mg myo-inositol, 0.38 mg D-biotin, 2.5 mg folic acid, 0.03 mg cyanocobalamin, 2.8 mg retinol acetate, 0.1 mg cholecalciferol, 250 mg α -tocopherol acetate, 5 mg menadione sodium bisulphate by Sigma-Aldrich, Castle Hill, NSW, Australia and 100 mg Roche Rovimix E50, ⁹Mineral mix (TMV4) supplied per kilogram of feed: 117 mg CuSO₄·5H₂O, 7.19 mg KI, 1815 mg FeSO₄·7H₂O, 307 mg MnSO₄·H₂O, 659 mg ZnSO₄·7H₂O, 329 mg Na₂SeO₃, 47.7 mg CoSO₄·7H₂O by Sigma-Aldrich, Castle Hill, NSW, Australia, ¹⁰Roche Vitamins Australia, Frenchs Forest, NSW, Australia, ¹¹Sigma-Aldrich, Castle Hill, NSW, Australia, ¹² Degussa, Frankfurt, Germany, EO, *Echium* oil diet; RO, rapeseed oil diet; FO, fish oil diet, SFA, Saturated fatty acids; CMC, Carboxymethyl cellulose; SDA, Stearidonic acid, ALA, α -Linolenic acid; LA, Linoleic acid; GLA, γ -linolenic acid.

2.3.5 Lipid extraction and isolation

Whole carcass and faecal samples were freeze dried and extracted overnight using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single phase extraction using CHCl₃/MeOH/H₂O (1:2:0.8, v/v/v), followed by phase separation to yield a total lipid extract (TLE).

An aliquot of the TLE was trans-methylated in methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 2 h at 100°C. After addition of MilliQ water (1 ml), the mixture was extracted with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). Samples with an internal injection standard (19:0 FAME) added were analysed by gas chromatography (GC) using an Agilent Technologies 7890B GC (Palo Alto, California, USA) equipped with an EquityTM-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 μ m film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683 B Series auto sampler. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 120°C. After injection, oven temperature was raised to 270°C at 10°C/min and finally to 310°C at 5°C/min. Peaks were quantified with Agilent Technologies ChemStation

software (Palo Alto, California, USA). GC results are typically subject to an error of up to $\pm 5\%$ of individual component area.

Individual components were identified by mass spectral data and by comparing retention time data with authentic and laboratory standards. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, Texas, USA). The GC was equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m \times 0.32 mm i.d.) of similar polarity to that described above. Helium was used as carrier gas, with operating conditions previously described (Miller et al., 2006).

2.3.6 *Fatty acid mass balance*

A whole body FAMB was performed on the n-3 biosynthetic pathway to compare individual fatty acid appearance or disappearance and the accretion of individual n-3 PUFA as previously described (Turchini et al., 2007).

2.3.7 *Statistical analysis*

Values are reported as mean \pm standard error of the mean (SEM). Normality and homogeneity of variance were confirmed and percentage data were arcsine transformed prior to analysis. Comparison between treatments for fatty acid, growth performance, osmolality, condition factor and mass balance means was by one way ANOVA followed by multiple comparison using Tukey-Kramer HSD wherever applicable. Significance was accepted at probabilities of 0.05 or less. Statistical analysis was performed using SPSS for Windows version 16.0.

2.4 RESULTS

2.4.1 Parr-smolt transformation

There were no significant differences ($P > 0.05$) in blood plasma osmolality of fish fed on all diets 7 days post transfer to seawater. Mean condition factor (K) for all groups was similar during the short day period, but shortly after switching to continuous light RO and EO fish displayed higher K compared to FO fish prior to seawater transfer ($P < 0.05$) (Fig. 2.1). After seawater transfer, elevated K was observed particularly for RO fish. The pattern was similar for all groups and was typical for smolting fish, with a steady drop in K after onset of continuous light which carried on for 28 d in seawater before increasing thereafter.

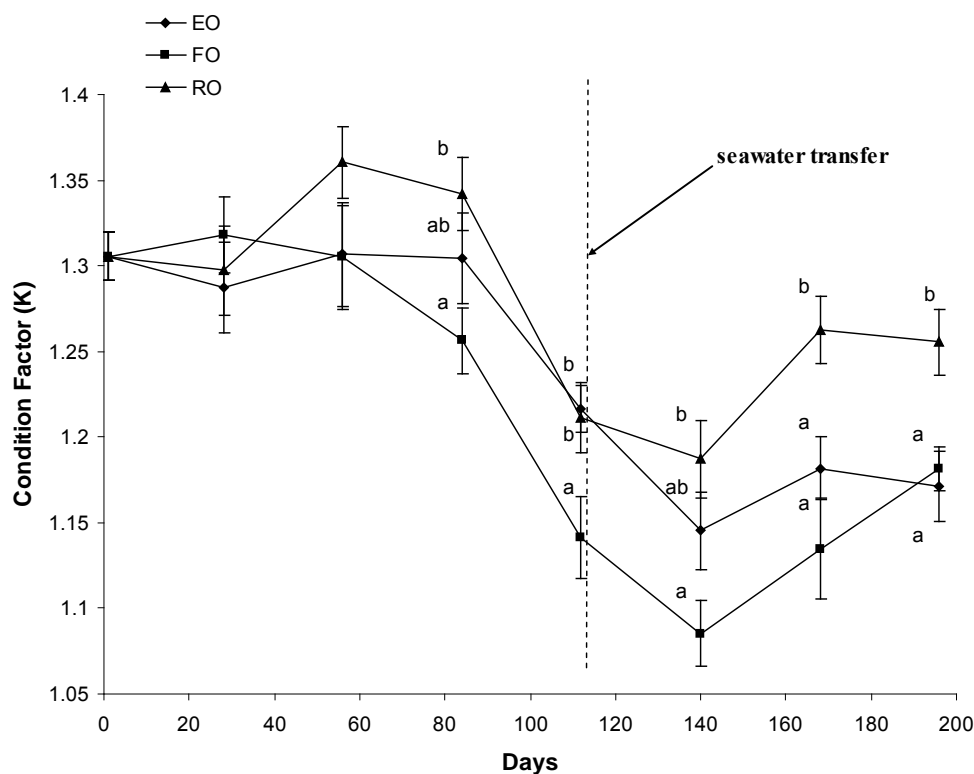


Figure 2.1: Condition factor (K) of Atlantic salmon fed on different oil sources from parr to smolt. Values (mean \pm SEM; $n = 16$) represents K of Atlantic salmon fed diets containing *Echium* oil (EO), Fish oil (FO) and Rapeseed oil (RO). Different letters at each time interval represent significant differences ($P < 0.05$) in K between diets.

2.4.2 Growth

Growth (final weight, weight gain, SGR) and efficiency was higher ($P < 0.05$) for RO fish compared to EO fish in freshwater, whereas, FO fish did not differ significantly ($P > 0.05$) in performance and efficiency from the other two treatments. Feed consumption (FC) and survival were not significantly different ($P > 0.05$) between treatments (Table 2.2).

Growth (final weight, weight gain, SGR) was higher for RO fish compared to fish fed on EO and FO diets in seawater, with no difference ($P > 0.05$) in growth performance for EO and FO fish (Table 2.2). Survival was higher in RO fish compared to FO and EO fish.

2.4.3 Chemical composition

During the freshwater phase, feeds had no significant effect on carcass dry matter (DM) (pooled mean \pm SEM, 326.1 ± 2.5 g/kg), crude protein (159.5 ± 1.1 g/kg, WW), total lipid (124.4 ± 3.4 g/kg, WW) or ash (26.3 ± 0.5 g/kg, WW). In the seawater phase, feeds had no significant effect on carcass crude protein (pooled mean \pm SEM, 146.6 ± 1.0 g/kg, WW), total lipid (120.2 ± 2.7 g/kg, WW) or ash (25.4 ± 0.4 g/kg, WW). Significant differences for carcass DM ($P < 0.05$) were obtained (EO = 314.4 ± 3.3^a ; FO = 320.8 ± 4.6^{ab} ; RO = 328.0 ± 3.6^b) with EO fish having a lower DM than RO fish.

FO fish had higher whole carcass EPA and DHA content than either EO or RO fish in freshwater. EO fish had significantly higher ($P < 0.01$) SDA, α -linolenic acid (ALA; 18:3n-3) and eicosatetraenoic acid (ETA; 20:4n-3) compared to the other two diets (Table 2.3). Total n-3 for EO fish was comparable to FO fish and the total

PUFA was higher for EO fish compared to FO fish, whereas for RO fish, both total n-3 and total PUFA were lowest ($P < 0.01$). The n-3: n-6 ratios for fed fish were in the order FO > EO > RO.

FO fish had higher whole carcass EPA and DHA content than either EO or RO fish in seawater. EO fish had significantly higher ($P < 0.01$) ALA, SDA and ETA compared to the other two diets (Table 2.4). There was significantly higher ($P < 0.05$) EPA and docosapentaenoic acid (DPA; 22:5 n-3) in EO fish compared to RO fish. Total n-3 and total PUFA were higher ($P < 0.01$) for EO fish compared to FO and RO fish. The n-3: n-6 ratios for FO, EO and RO fish were in the order FO > EO > RO. Total FA was significantly higher ($P < 0.05$) in RO fish compared to EO and FO fish.

Table 2.2: Growth and efficiencies of Atlantic salmon fed EO, FO and RO diets

	Diet	Initial weight (g)			Final weight (g)			Weight gain (g)			SGR (%/d)			FC (g)			FER (g/g)			Survival (%)		
Freshwater	EO	26.1	±	0.5	96.3	±	0.9 ^a	70.2	±	0.9 ^a	1.2	±	0.0 ^a	73.5	±	1.1	1.0	±	0.0 ^a	93.8	±	1.4
	FO	25.7	±	0.3	103.5	±	2.7 ^{ab}	77.9	±	2.8 ^{ab}	1.2	±	0.0 ^{ab}	76.8	±	1.5	1.0	±	0.0 ^{ab}	94.7	±	0.8
	RO	26.4	±	0.2	109.4	±	3.0 ^b	83.1	±	3.0 ^b	1.3	±	0.0 ^b	79.1	±	1.6	1.1	±	0.0 ^b	95.5	±	0.0
Seawater	EO	96.3	±	0.9 ^a	204.4	±	2.4 ^a	108.1	±	1.8 ^a	0.9	±	0.0 ^a	134.6	±	4.3 ^a	0.8	±	0.0	81.3	±	1.2 ^a
	FO	103.5	±	4.3 ^{ab}	223.0	±	11.0 ^a	119.5	±	6.7 ^a	0.9	±	0.0 ^a	143.2	±	2.0 ^{ab}	0.8	±	0.0	81.9	±	2.1 ^a
	RO	109.4	±	3.0 ^b	252.8	±	8.7 ^b	143.4	±	5.9 ^b	1.0	±	0.0 ^b	159.7	±	5.3 ^b	0.9	±	0.0	92.7	±	2.6 ^b

Values are mean ± SEM, $n = 3$ (FO) and $n = 4$ (EO and RO). Means in a column belonging to either freshwater or seawater with different superscript letters were significantly different ($P < 0.05$). EO, *Echium* oil diet; FO, fish oil diet; RO, rapeseed oil diet.

Table 2.3: Fatty acid content (mg/g) of whole carcasses of Atlantic salmon parr fed EO, FO and RO diets

FA	Initial		EO		FO		RO		<i>f</i>
14:0	11.3	± 0.3 ^b	1.9	± 0.2 ^a	9.6	± 0.8 ^b	1.9	± 0.2 ^a	100.5
16:0	38.8	± 0.6 ^b	24.7	± 0.6 ^a	40.4	± 1.5 ^b	22.4	± 1.1 ^a	68.8
18:0	11.4	± 0.0 ^{ab}	13.0	± 0.0 ^b	11.7	± 0.5 ^b	9.4	± 0.4 ^a	18.1
Other SFA ¹	5.1	± 0.1 ^b	1.3	± 0.2 ^a	4.8	± 0.3 ^b	1.7	± 0.2 ^a	59.1
Total SFA	66.5	± 1.2 ^b	40.9	± 1.1 ^a	66.5	± 2.6 ^b	35.5	± 1.5 ^a	81.7
16:1n-7c	21.6	± 0.3 ^b	5.8	± 0.3 ^a	21.1	± 0.7 ^b	5.7	± 0.5 ^a	295.2
18:1n-9c	41.8	± 1.4 ^a	44.3	± 1.0 ^a	35.6	± 1.0 ^a	107.7	± 5.1 ^b	111.7
18:1n-7c	11.1	± 0.2 ^b	4.7	± 0.1 ^a	10.8	± 0.4 ^b	9.7	± 0.4 ^b	88.4
20:1n-9c	7.8	± 0.7 ^b	3.6	± 0.2 ^a	4.1	± 0.2 ^a	4.7	± 0.2 ^a	27.5
Other MUFA ²	7.9	± 0.3 ^c	2.7	± 0.2 ^a	5.4	± 0.2 ^b	3.3	± 0.3 ^a	46.2
Total MUFA	90.2	± 2.3 ^b	61.0	± 1.5 ^a	77.0	± 1.8 ^{ab}	131.1	± 5.8 ^c	68.8
18:3n-3 ALA	0.5	± 0.3 ^a	24.7	± 1.1 ^c	2.3	± 0.3 ^a	9.9	± 0.6 ^b	169.3
18:4n-3 SDA	3.4	± 0.4 ^a	18.5	± 1.0 ^b	5.1	± 0.2 ^a	4.2	± 0.3 ^a	120.3
20:4n-3 ETA	2.6	± 0.3 ^b	3.1	± 0.2 ^b	1.2	± 0.3 ^a	1.1	± 0.1 ^a	27.6
20:5n-3 EPA	12.6	± 1.5 ^b	5.9	± 0.4 ^a	21.8	± 1.0 ^c	4.5	± 0.3 ^a	151.5
22:5n-3 DPA	5.4	± 0.5 ^b	2.8	± 0.2 ^a	8.9	± 0.4 ^c	1.7	± 0.3 ^a	120.7
22:6(n-3) DHA	17.8	± 1.2 ^b	8.6	± 0.7 ^a	20.4	± 0.8 ^b	7.4	± 0.5 ^a	78.5
Other n-3 ³	1.0	± 0.0 ^b	0.0	± 0.0 ^a	1.5	± 0.1 ^c	0.1	± 0.1 ^a	212.7
Total n-3	43.5	± 4.3 ^b	63.6	± 2.9 ^c	61.2	± 2.6 ^c	28.9	± 1.4 ^a	46.9
18:2n-6 LA	7.7	± 0.9 ^a	31.4	± 1.1 ^b	11.1	± 0.5 ^a	31.1	± 1.2 ^b	114.5
18:3n-6 GLA	0.0	± 0.0 ^a	11.2	± 0.5 ^c	0.0	± 0.0 ^a	3.0	± 0.2 ^b	227.3
20:3n-6	0.0	± 0.0 ^a	3.5	± 0.1 ^c	0.1	± 0.1 ^a	2.3	± 0.1 ^b	122.3
20:4n-6 ARA	1.6	± 0.1 ^b	0.9	± 0.1 ^a	2.2	± 0.1 ^c	1.2	± 0.1 ^{ab}	28.7
Other n-6 ⁴	0.9	± 0.1	0.2	± 0.1	0.4	± 0.2	0.8	± 0.3	
Total n-6	10.3	± 0.9 ^a	47.1	± 1.7 ^c	13.9	± 0.6 ^a	38.5	± 1.6 ^b	121.1
Other PUFA ⁵	1.7	± 0.2 ^b	0.2	± 0.1 ^a	2.6	± 0.1 ^c	0.2	± 0.1 ^a	98.2
Total PUFA	55.5	± 5.4 ^a	110.9	± 4.5 ^c	77.7	± 3.1 ^b	67.5	± 2.3 ^{ab}	39.7
n-3:n-6	4.2	± 0.0 ^c	1.3	± 0.0 ^b	4.4	± 0.2 ^c	0.8	± 0.0 ^a	273.5
Total FA	212.2	± 3.3	212.8	± 6.4	221.2	± 4.6	234.1	± 8.9	

EO, *Echium* oil diet; FO, fish oil diet; RO, rapeseed oil diet.

f, Mean sum of squares.

^{a,b,c} Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; *df*=3, *P* <0.01.

¹ Includes 15:0, 17:0, 20:0, 22:0 and 24:0.

² Includes 16:1n-9, 16:1n-5, 18:1n-5, 20:1n-7, 22:1n-9, 22:1n-11 and 24:1n-9.

³ Includes 21:5n-3 and 24:6n-3.

⁴ Includes 20:2n-6, 22:4n-6 and 24:5n-6.

⁵ Includes 16:2n-4, 16:3n-4 and 18:2n-9.

Table 2.4: Fatty acid content (mg/g) of whole carcasses of Atlantic salmon smolt fed EO, FO and RO diets

FA	EO			FO			RO			F
14:0	1.1	±	0.1 ^a	6.4	±	0.7 ^b	1.3	±	0.1 ^a	64.9
16:0	22.1	±	0.1 ^a	33.6	±	0.9 ^b	19.1	±	0.5 ^a	99.0
18:0	12.5	±	0.4 ^b	9.2	±	0.3 ^a	8.6	±	0.2 ^a	45.5
Other SFA ¹	0.9	±	0.1 ^a	4.0	±	0.3 ^c	2.2	±	0.1 ^b	73.7
Total SFA	36.8	±	1.3 ^b	53.3	±	1.5 ^c	31.2	±	0.8 ^a	80.4
16:1n-7c	2.8	±	0.2 ^a	16.9	±	0.4 ^b	3.2	±	0.2 ^a	776.8
18:1n-9c	45.5	±	1.3 ^b	33.4	±	1.5 ^a	123.4	±	2.0 ^c	870.2
18:1n-7c	3.6	±	0.1 ^a	9.6	±	0.3 ^b	10.0	±	0.2 ^b	252.5
20:1n-9c	2.8	±	0.1 ^a	5.1	±	0.5 ^b	4.6	±	0.2 ^b	17.6
Other MUFA ²	2.0	±	0.2 ^a	6.5	±	0.6 ^b	1.5	±	0.2 ^a	55.8
Total MUFA	56.7	±	1.7 ^a	71.4	±	3.0 ^b	142.6	±	2.4 ^c	420.5
18:3n-3 ALA	31.1	±	1.4 ^c	2.5	±	0.3 ^a	10.4	±	0.2 ^b	248.7
18:4n-3 SDA	22.6	±	1.4 ^b	4.3	±	0.2 ^a	4.1	±	0.1 ^a	146.3
20:4n-3 ETA	3.0	±	0.2 ^b	0.8	±	0.2 ^a	0.6	±	0.1 ^a	70.4
20:5n-3 EPA	4.5	±	0.1 ^b	21.5	±	1.0 ^c	2.6	±	0.1 ^a	405.6*
22:5n-3 DPA	1.7	±	0.2 ^b	7.9	±	0.6 ^c	0.6	±	0.2 ^a	136.2
22:6n-3 DHA	5.1	±	0.2 ^a	17.9	±	0.5 ^b	4.1	±	0.2 ^a	575.5
Other n-3 ³	0.0	±	0.0 ^a	1.2	±	0.2 ^b	0.0	±	0.0 ^a	78.1
Total n-3	68.0	±	3.3 ^c	56.1	±	2.5 ^b	22.5	±	0.5 ^a	105.2
18:2n-6 LA	38.2	±	1.4 ^b	10.3	±	0.3 ^a	39.8	±	0.5 ^b	266.0
18:3n-6 GLA	13.9	±	0.7 ^c	0.1	±	0.1 ^a	3.8	±	0.1 ^b	256.8
20:3n-6	3.7	±	0.2 ^c	0.0	±	0.0 ^a	2.8	±	0.1 ^b	159.1
20:4n-6 ARA	0.4	±	0.1 ^a	2.1	±	0.1 ^c	1.1	±	0.1 ^b	46.3
Other n-6 ⁴	0.5	±	0.1 ^a	0.4	±	0.1 ^a	1.1	±	0.2 ^b	8.1
Total n-6	56.7	±	2.4 ^c	12.9	±	0.6 ^a	48.7	±	0.6 ^b	188.3
Other PUFA ⁵	0.0	±	0.0 ^a	2.0	±	0.1 ^b	0.0	±	0.0 ^a	364.4
Total PUFA	124.7	±	5.7 ^b	71.0	±	3.1 ^a	71.1	±	0.8 ^a	65.6
n-3:n-6	1.2	±	0.0 ^b	4.4	±	0.1 ^c	0.5	±	0.0 ^a	788.5
Total FA	218.2	±	8.1 ^a	195.7	±	6.5 ^a	245.0	±	3.7 ^b	14.2*

EO, *Echium* oil diet; FO, fish oil diet; RO, rapeseed oil diet.

f, Mean sum of squares.

^{a,b,c} Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; $df=3$, $P < 0.01$, * $P < 0.05$.

¹ Includes 15:0, 17:0, 20:0, 22:0 and 24:0.

² Includes 16:1n-9, 16:1n-5, 18:1n-5, 20:1n-7, 22:1n-9, 22:1n-11 and 24:1n-9.

³ Includes 21:5n-3 and 24:6n-3.

⁴ Includes 20:2n-6, 22:4n-6 and 24:5n-6.

⁵ Includes 16:2n-4, 16:3n-4 and 18:2n-9.

2.4.4 Fatty acid mass balance

Biosynthesis of ETA and EPA was a higher ($P < 0.05$) for EO fish compared to both RO and FO fish, with no difference in the biosynthesis of DPA amongst all groups in freshwater (Table 2.5). DHA showed a positive mass balance (PMB) which was not different between EO and RO fish. The main negative mass balance (NMB)

in EO fish was for ALA which represented 69% of the net intake (Table 5) followed by SDA (42%). Total elongated/desaturated n-3 LC-PUFA products (ETA+EPA+DPA+DHA = 1249 $\mu\text{mol}/\text{fish}$) represented 15% of the combined NMB in ALA and SDA in EO fish (8414 $\mu\text{mol}/\text{fish}$). There was a NMB of 2327 $\mu\text{mol}/\text{fish}$ of ALA in RO fish which represented 63% of net intake (Table 2.5). A NMB of 1096 $\mu\text{mol}/\text{fish}$ (47%) for ALA was obtained in RO fish as a result of elongation and desaturation, out of which SDA biosynthesis represented 42% (464 $\mu\text{mol}/\text{fish}$). Total elongated/desaturated n-3 LC-PUFA products (ETA+EPA+DPA+DHA) in RO fish were 632 $\mu\text{mol}/\text{fish}$. In FO fish, the main NMB was for EPA, with a disappearance of around 66% of net intake (4264 $\mu\text{mol}/\text{fish}$), mainly due to β -oxidation as only 228 $\mu\text{mol}/\text{fish}$ was used for biosynthesis of DPA.

Higher biosynthesis of ETA ($P < 0.05$) occurred in EO fish compared to RO and FO fish in seawater. There were no significant differences ($P > 0.05$) in EPA, ETA and DHA between EO and RO fish, but negative values of EPA, ETA and DHA were obtained for RO fish (Table 2.5). In contrast, there was a PMB of all n-3 LC-PUFA along the n-3 pathway for EO fish (Table 2.5).

There was a NMB of 12836 $\mu\text{mol}/\text{fish}$ and 3472 $\mu\text{mol}/\text{fish}$ for ALA and SDA respectively for EO fish in seawater accounting for 73% and 51% of net intakes. Total elongated/desaturated products of n-3 LC-PUFA (ETA+EPA+DPA+DHA) were 696 $\mu\text{mol}/\text{fish}$ for EO fish representing only 4% of combined ALA and SDA NMB. There was a NMB of 74% (5468 $\mu\text{mol}/\text{fish}$) of ALA net intake from RO fish, with only 13% (689 $\mu\text{mol}/\text{fish}$) elongated/desaturated along the pathway largely as SDA (633 $\mu\text{mol}/\text{fish}$). There was no PMB of FA for FO fish in seawater and the main NMB was EPA (79% net intake) followed by DHA (52% net intake).

Higher accretion of $\Delta 5$ desaturated and elongated FA ($P < 0.05$) in EO fish compared to CO fish was observed in freshwater (Fig. 2.2). The accretion of elongated and desaturated FA could not be computed for RO and FO fish in seawater as a result of high NMB along the n-3 pathway.

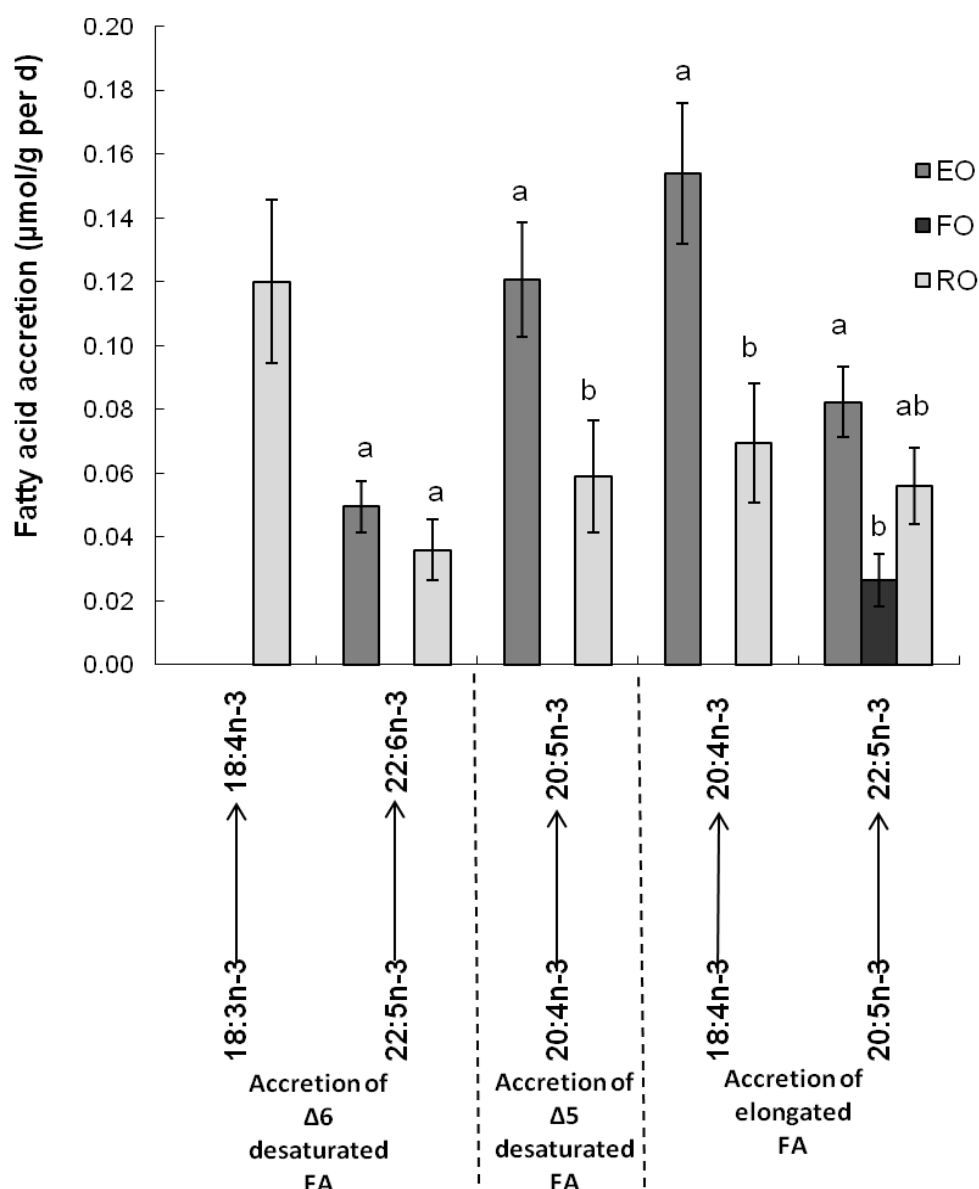


Figure 2.2: The individual appearance (accretion) of $\Delta 5$ and $\Delta 6$ desaturated and elongated fatty acids in whole carcass of Atlantic salmon parr fed on different oil sources in freshwater. Values are mean \pm SEM, $n = 6$ for FO and $n = 8$ for EO and RO. Different letters represent significant differences ($P < 0.05$) in FA accretion between diets. EO, *Echium* oil diet; FO, fish oil diet; RO, rapeseed oil diet.

Higher accretion of $\Delta 5$ desaturated and elongated FA ($P < 0.05$) in EO fish compared to CO fish was observed in freshwater (Fig. 2.2). The accretion of elongated and desaturated FA could not be computed for RO and FO fish in seawater as a result of high NMB along the n-3 pathway.

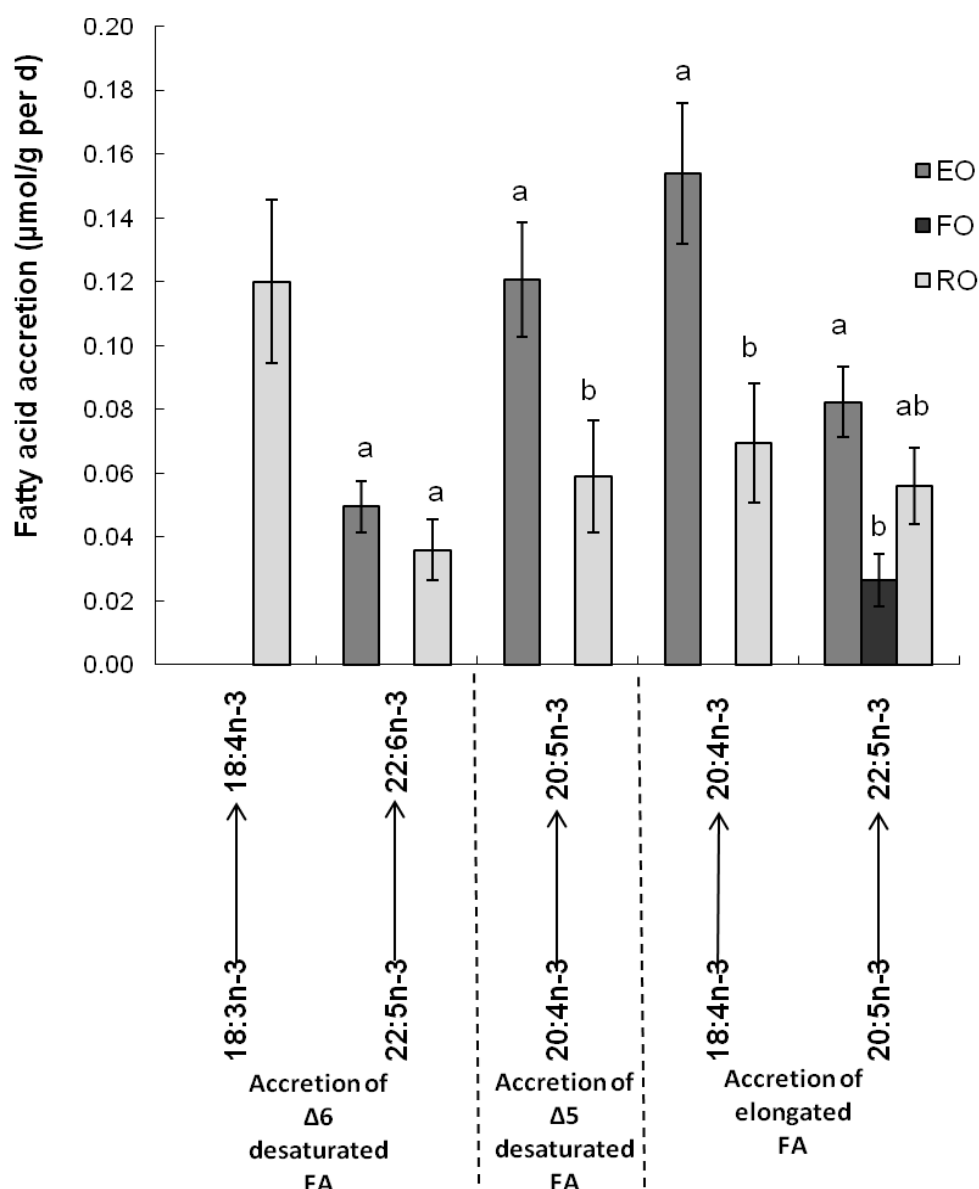


Figure 2.2: The individual appearance (accretion) of $\Delta 5$ and $\Delta 6$ desaturated and elongated fatty acids in whole carcass of Atlantic salmon parr fed on different oil sources in freshwater. Values are mean \pm SEM, $n = 6$ for FO and $n = 8$ for EO and RO. Different letters represent significant differences ($P < 0.05$) in FA accretion between diets. EO, *Echium* oil diet; FO, fish oil diet; RO, rapeseed oil diet.

Table 2.5: Fatty acid mass balance (μmol/fish) for n-3 PUFA series in whole carcasses of Atlantic salmon fed EO, FO and RO diets

		ALA (18:3n-3)		SDA (18:4n-3)		ETA (20:4n-3)		EPA (20:5n-3)		DPA (22:5n-3)		DHA (22:6n-3)	
Freshwater	Net Intake												
	EO	9813.0	± 144.5 ^c	3792.4	± 55.8 ^c	13.7	± 0.2 ^b	54.7	± 0.8 ^a	0.0	± 0.0 ^a	72.8	± 1.1 ^a
	FO	498.8	± 10.0 ^a	1089.8	± 21.8 ^b	272.5	± 5.4 ^c	6504.1	± 129.8 ^b	705.5	± 14.1 ^b	2067.9	± 41.3 ^b
	RO	3697.4	± 75.8 ^b	34.6	± 0.7 ^a	0.0	± 0.0 ^a	217.9	± 4.5 ^a	0.0	± 0.0 ^a	130.5	± 2.7 ^a
	Accumulation												
	EO	3006.2	± 199.6 ^c	2185.4	± 116.4 ^b	282.8	± 26.0 ^b	369.4	± 55.5 ^a	263.4	± 20.3 ^a	474.7	± 73.2 ^a
	FO	280.2	± 48.2 ^a	555.3	± 46.0 ^a	69.8	± 56.3 ^a	2240.2	± 200.2 ^b	933.1	± 64.4 ^b	1783.8	± 155.0 ^b
	RO	1370.7	± 74.0 ^b	498.9	± 46.8 ^a	72.2	± 15.0 ^a	247.7	± 46.3 ^a	200.3	± 18.6 ^a	459.4	± 80.9 ^a
	Appearance/disappearance												
	EO	-6806.8	± 151.7 ^a	-1607.0	± 110.7 ^a	269.1	± 25.0 ^c	314.7	± 55.6 ^c	263.4	± 20.3 ^a	401.8	± 72.9 ^b
Seawater	FO	-218.7	± 45.3 ^c	-534.5	± 29.4 ^b	-202.7	± 51.7 ^a	-4264.0	± 119.0 ^a	227.6	± 66.2 ^a	-284.1	± 143.7 ^a
	RO	-2326.8	± 120.9 ^b	464.4	± 47.5 ^c	72.2	± 15.0 ^b	29.7	± 50.6 ^b	200.3	± 18.0 ^a	328.9	± 82.9 ^b
	Net Intake												
	EO	17514.7	± 599.6 ^c	6808.5	± 233.1 ^c	0.0	± 0.0 ^a	104.0	± 3.6 ^a	0.0	± 0.0 ^a	140.1	± 4.8 ^a
	FO	907.4	± 7.4 ^a	1973.5	± 16.0 ^b	470.5	± 3.8 ^b	11725.3	± 95.2 ^c	1276.4	± 10.4 ^b	3579.4	± 29.1 ^c
	RO	7398.0	± 243.5 ^b	69.8	± 2.3 ^a	0.0	± 0.0 ^a	446.1	± 14.7 ^b	0.0	± 0.0 ^a	266.8	± 8.8 ^b
	Accumulation												
	EO	4678.9	± 518.3 ^c	3336.1	± 538.2 ^b	337.2	± 39.4 ^b	330.9	± 42.3 ^a	112.6	± 30.2 ^a	159.7	± 79.5 ^a
	FO	356.1	± 107.5 ^a	455.6	± 97.4 ^a	63.9	± 50.6 ^a	2485.5	± 449.5 ^b	725.3	± 250.0 ^b	1705.1	± 348.2 ^b
	RO	1930.4	± 180.8 ^b	702.4	± 96.4 ^a	56.0	± 28.9 ^a	182.4	± 79.5 ^a	-72.5	± 35.9 ^a	208.3	± 89.7 ^a
	Appearance/disappearance												
	EO	-12835.9	± 676.1 ^a	-3472.3	± 573.8 ^a	337.2	± 39.4 ^c	226.9	± 39.6 ^c	112.6	± 30.2 ^b	19.6	± 75.5 ^b
	FO	-551.3	± 101.6 ^c	-1517.8	± 86.9 ^b	-406.6	± 47.2 ^a	-9239.8	± 382.0 ^a	-551.1	± 241.6 ^a	-1874.3	± 320.5 ^a
	RO	-5467.6	± 104.8 ^b	632.6	± 94.2 ^c	56.0	± 28.9 ^b	-263.7	± 68.1 ^b	-72.5	± 35.9 ^b	-58.5	± 85.7 ^b

Values are mean ± SEM, n = 6 and n = 9 for FO in freshwater and seawater respectively, n = 8 and n = 12 for EO and RO in freshwater and seawater respectively. Means in a column belonging to either freshwater or seawater with different superscript letters were significantly different ($P < 0.05$). EO, *Echium* oil diet; FO, fish oil diet; RO, rapeseed oil diet.

2.5 DISCUSSION

2.5.1 *Growth and parr-smolt transformation*

The transfer and growth of fish in seawater was a key element of this experiment and both the condition factor (K) (Fig. 2.1) and osmolality values for all groups were indicative of smolted fish. Feeding Atlantic salmon a diet of 100% RO had a positive impact on growth of fish over the duration of the experiment. Alterations in lipid metabolism are regarded as an integral part of the parr-smolt transformation and a VO diet might be better suited for Atlantic salmon to adapt to seawater since its fatty acid composition more closely resembles those of fish from the wild (Bell et al., 1994). The importance of dietary VO during smoltification might be multiple: higher osmoregulatory capacity (Bell et al., 1997; Tocher et al., 2000), increased growth (Bendiksen et al., 2003) and acting as a protection barrier against translocation of pathogens (Jutfelt et al., 2007). In agreement with previous findings on the beneficial effects of VO on fish undergoing smoltification, RO fish showed higher growth and survival indicating that the RO fish were better prepared for transition in seawater than EO and FO fish. To the best of my knowledge, in all previous studies involving complete substitution of FO by VO in Atlantic salmon undergoing parr-smolt transformation, fishmeal contributed some n-3 LC-PUFA to the diet with EPA and DHA composition ranging from 1% to 4% of total fatty acids (Bell et al., 1997; Tocher et al., 2000; Bendiksen et al., 2003). In this study, defatted fishmeal was used and only trace amounts of n-3 LC-PUFA were present in the VO diets with EPA and DHA composition ranging from 0.2% to 0.5% of total fatty acids (0.3-0.9 g/kg, Table 2.1). An important finding of this present study in relation to parr-smolt transformation was that fish fed exclusively on VO diets (EO or RO) successfully smolted. This finding could prove to be important for feed formulation

in the context of FO substitution in Atlantic salmon in freshwater and also extends to fishmeal replacement in the freshwater life cycle of Atlantic salmon.

2.5.2 n-3 Fatty acid metabolism – freshwater phase

The prime objective was to test whether feeding Atlantic salmon a diet rich in SDA from parr to smolt would result in higher biosynthesis of n-3 LC-PUFA. In freshwater, EO fish had higher ETA compared to fish fed the other diets (Table 2.3). Atlantic salmon parr towards the end of the freshwater period were at an important phase in their life cycle, preparing for the transfer to seawater. This critical period for the fish is accompanied by an increase in desaturation and elongation activity along both the n-3 and n-6 pathways for the production of LC-PUFA (Bell et al., 1997; Tocher et al., 2000). Therefore if provided with enough substrate (ALA), fish can meet their EPA and DHA requirements as observed in RO fish, and the presence of SDA in the EO diet, which is a precursor for EPA and allows bypassing of the initial $\Delta 6$ desaturase enzyme (Miller et al., 2007; 2008b), has enabled higher biosynthetic activity along the n-3 LC-PUFA pathway resulting in an increase in ETA. In a previous study on Atlantic salmon parr fed an EO diet, comparable levels of EPA and DHA in muscle were obtained to FO fed fish (Miller et al., 2007). In this present study, similar result was not obtained. However, in the present study, the condition factor was higher because the diet contained 200 g/kg total lipids compared to 129 g/kg in the previous study. Therefore due to the lower dietary lipid level, there was a reduction in TAG proportion relative to polar lipids (PL) in the white muscle which might explain the retention of DHA (Miller et al., 2007). Furthermore, the previous study was conducted over a shorter duration (42 days) which coincided with the critical parr-smolt transformation period characterised by an increase in desaturation and elongation activity.

A point of focus was the biosynthetic activity along the n-3 pathway and the FAMB approach was used to verify our hypothesis. For the EO fish, ETA and EPA biosynthesis was higher compared to RO fish due to the presence of high SDA (9.2% of total FA) in EO diet. In contrast, for RO fish, ALA had to be desaturated to SDA, adding an extra step along the pathway at the cost of 42% of total elongation and desaturation products. As a result there was a two fold increase in total n-3 LC-PUFA biosynthesis (1249 $\mu\text{mol}/\text{fish}$ v/s 631 $\mu\text{mol}/\text{fish}$) in EO fish. There was no difference in DHA biosynthesis between EO and RO fish, hereby underlying the importance of DHA in fish undergoing smoltification and life in seawater. Previous research has demonstrated that in Atlantic salmon undergoing parr-smolt transformation, there was an increase in DHA in gill and liver PL in fish fed on VO diets (Bell et al., 1997; Tocher et al., 2000). In other studies (Bell et al., 2001, 2002), there was preferential deposition and retention of DHA in muscle lipids irrespective of the concentration in the diet which was attributed to the specificity of fatty acyl transferase enzymes towards incorporation of DHA into flesh TAG and PL.

FO fish in freshwater showed some degree of biosynthetic activity especially for DPA and while not neglecting the fact that EPA and DHA could have been produced, it might have been masked by the high dietary presence of these FA. This masking is regarded as a shortcoming associated with the fatty acid mass balance approach (Turchini et al., 2007, 2009; Francis et al., 2009). Consequently when computing the last step of the method, it was not possible to detect any increment of FA as a result of $\Delta 5$, $\Delta 6$ desaturases and elongase enzymes except for the conversion of EPA to DPA (Fig. 2.2) with a PMB obtained for DPA. Other limitations may occur; the computation of the FAMB proceeds only in the direction of its specific pathway, therefore, it does not take into account possible FA chain-shortening such as

retro conversion of DHA to DPA or the β -oxidation of FA previously elongated and desaturated (Turchini et al., 2007, 2009). The production of eicosanoids, resolvins and protectins from their precursor's arachidonic acid, EPA and DHA are also not quantified. However, the production of these functional metabolites is minimal, probably having little impact on the total FAMB (Turchini et al., 2007, 2009). In rats, the production of eicosanoids, as measured by their urinary excretion, does not exceed 1 μ g/d (Hansen & Jensen, 1983). Most of these metabolites are generally involved in inflammatory processes and are either potent pro- or anti-inflammatory at nanomolar concentrations and their production is measured in minute amounts (ng/mg of protein) in tissues (Marcheselli et al., 2003; Hudert et al., 2006). Arguably, the FAMB might be more limited with respect to production of these functional metabolites in cases of infected or injured fish. Similar limitations also occur with other methods employing labelled FA to assess FA metabolism in fish (Turchini et al., 2009). As use of the FAMB approach expands, including via incorporation of measurement of metabolites present at low abundance, the method may be further fine tuned.

Similarly, for EO fish, the high presence of SDA in the diet might have masked its desaturation from ALA, therefore any accretion of Δ 6 desaturated SDA could not be obtained (Fig. 2.2). Hence, when assessing the biosynthetic activity along the n-3 pathway, this method might be best suited when comparing between oils with ALA as the main precursor and very low amounts of other n-3 PUFA. In freshwater, ALA was the main FA β -oxidised in RO fish (33% of net intake), while in EO fish, 69% and 42% of ALA and SDA net intake showed a NMB. The 42% SDA NMB equated to 1607 μ mol/fish which was greater than the total elongated/desaturated products (1249 μ mol/fish). Therefore theoretically 78% of SDA NMB was biosynthesised along the n-3 pathway and dietary ALA did not

contribute to any n-3 LC-PUFA biosynthesis in EO fish. Since some ALA might have been elongated to SDA and then further metabolised, as mentioned above, the high dietary amount of SDA might have masked this step.

In FO fish, 66% of EPA net intake showed a NMB mainly through β -oxidation. It has been well documented that excess dietary EPA is readily β -oxidised (Stubhaug et al., 2007; Francis et al., 2009; Turchini et al., 2009). Moreover, it has been shown that at around seawater transfer there is an increase in β -oxidation capacity in Atlantic salmon liver and muscle (Tocher, 2003; Stubhaug et al., 2006) which would explain the apparent β -oxidation of EPA. The FAMB approach was developed to determine enzymatic activity (Turchini et al., 2007), however, since the enzyme activity is usually measured over a limited incubation time, it was proposed to either report any FA accretion as an indication of enzymatic activity (Turchini et al., 2009) or apparent enzyme activity (Francis et al., 2009). In this study, the accretion of certain FA could not be computed due to the masking effect of the FA present in high amounts in the diet. However, in the freshwater phase, it was confirmed that in EO fish there was higher n-3 LC-PUFA production as a result of higher accretion of desaturated and elongated FA (Fig. 2.2).

2.5.3 n-3 Fatty acid metabolism – seawater phase

The same scenario as in freshwater was observed in seawater for whole carcass fatty acid content, with fish fed on FO diet having higher amounts of EPA and DHA (Table 2.4). However between the VO diets, the presence of SDA in EO fed fish in seawater resulted in greater n-3 LC-PUFA biosynthesis since higher ETA, EPA and DPA were accumulated. This observation was confirmed through the fatty acid mass balance (Table 2.5), where positive values were obtained at all levels of

desaturation/elongation along the n-3 pathway leading to a net gain of 696 $\mu\text{mol}/\text{fish}$ in total n-3 LC-PUFA for EO fish compared to a net loss (-439 $\mu\text{mol}/\text{fish}$) for RO fed fish. Yet again the presence of SDA in the EO diet resulted in these differences because of the extra step involved in producing SDA in RO fish at the expense of 633 $\mu\text{mol}/\text{fish}$ (Table 2.5). In a similar study (Miller et al., 2008b), it was found that an EO diet promoted an increase in elongase and $\Delta 5$ desaturase gene expression in Atlantic salmon smolt when compared to fish fed a FO diet and that the increase in activity lead to higher EPA in liver compared to fish fed a RO diet.

It was evident that the biosynthetic activity along the n-3 pathway was negligible in seawater for all three diets (Table 2.5). This observation has been previously documented, whereby marine fish cannot convert dietary ALA from VO sources to EPA and DHA at a physiologically significant rate (Sargent et al., 2002; Tocher, 2003) due to evolutionary consequence of a natural diet rich in n-3 LC-PUFA (Sargent et al., 2002). *E x vivo* approaches were used to assess n-3 biosynthetic capacity of Atlantic salmon in isolated hepatocytes in previous studies (Bell et al., 1997; Tocher et al., 1997, 2000) and showed lower hepatic desaturation of ALA to n-3 LC-PUFA in Atlantic salmon post-smolts compared to parr. The *in vivo* approach in this present study has also shown different biosynthetic capacity for Atlantic salmon in fresh and seawater. Due to the low n-3 biosynthetic activity at the cellular level of key tissues of Atlantic salmon in seawater and the NMB of substantial amounts of FA through β -oxidation, any significant accretion of n-3 LC-PUFA could not be detected when examined at the whole organism level in this study. The whole carcass was investigated in this study, therefore it will also be important to examine the individual tissues (muscle and liver) in future studies.

In seawater fish, the FAMB was characterized by high NMB of specific substrates mainly due to β -oxidation; ALA for RO fish (74% net intake), ALA and SDA for EO fish (73% and 51% net intake), EPA and DHA for FO fish (79% and 52% net intake). While there is a preferential order of FA for β -oxidation this is subservient to β -oxidation of excess FA (Turchini et al., 2009). ALA has been shown to be readily β -oxidised (Torstensen and Stubhaug, 2004; Stubhaug et al., 2005) in Atlantic salmon while SDA in EO fish and EPA in FO fish were probably supplied surplus to requirements from their respective diets. In general, immediately after seawater transfer, the feed intake of Atlantic salmon smolt is reduced due to stress and this period is accompanied by a reduction in condition (K) as fish use their lipid stores as an energy source. Therefore it is very likely that fish were initially using their lipid reserves in seawater which also contributed to the large NMB in FA.

The use of FO can be regarded as a rather wasteful practice due to the β -oxidation of substantial amounts of EPA and DHA; however, high amounts were still accumulated and resulted in the observed 3-5 fold difference compared to fish fed on VO diets. It should be stressed that from a human health perspective, the SDA enriched oil might be a more suitable substitute due to the improved n-3 :n-6 ratio, high levels of total n-3 (mostly as ALA and SDA) and total PUFA. Furthermore, high intake of SDA, from genetically modified soybean oil, increased the n-3 index and lowered risk of cardiac events in humans (Harris et al., 2008). EO in our study was used as model oil due to its naturally high SDA, but the use of EO is currently uneconomic as a substitute for FO (Miller et al., 2008a). Nevertheless, plant genomics research is underway to increase synthesis of SDA in commercially viable oil seed plants and also to further improve the n-3 :n-6 ratio (Ursin, 2003). In the near future such plant oils, including those containing EPA and DHA (Miller et al., 2008; Naylor

et al., 2009), might be commercially available and could be suitable for FO substitution in the diet of Atlantic salmon (Graham et al., 2004; Venegas-Caleron et al., 2010).

2.6 CONCLUSION

Several major findings were drawn from this study. Firstly, complete substitution of FO with both EO and RO in diets led to successful parr-smolt transformation without any additional input of n-3 LC-PUFA. Secondly, in freshwater, both RO and EO fish were able to biosynthesize n-3 LC-PUFA to meet their requirements, and the presence of SDA in the EO diet resulted in higher n-3 LC-PUFA biosynthesis. Thirdly, in seawater the n-3 LC-PUFA biosynthetic activity was non-existent at the whole body level for RO and FO fish, whereas some n-3 LC-PUFA biosynthesis occurred in EO fish probably as a result of the long feeding history on SDA, but to a lesser extent than in freshwater fish. In addition, the FAMB approach has been a useful tool to assess FA metabolism at the whole body level in this study, although further research is required to fine tune the method. Finally, although an EO diet increased the n-3 LC-PUFA biosynthesis, EPA and DHA content in both fresh and seawater fish was still lower compared to FO diet. However, due to higher ALA, SDA and total n-3 PUFA obtained in fish, use of oils enriched with SDA in aquafeeds would be more beneficial from a consumer perspective compared to conventional VO.

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CHAPTER 3

**An extended feeding history with a stearidonic acid enriched diet
from freshwater to seawater increases n-3 long-chain
polyunsaturated fatty acids biosynthesis in white muscle and liver of
Atlantic salmon (*Salmo salar* L.).**

Adapted from Codabaccus, M.B., Bridle, A.R., Nichols, P.D., Carter, C.G., 2011. An extended feeding history with a stearidonic acid enriched diet from freshwater to seawater increases n-3 long-chain polyunsaturated fatty acids biosynthesis in white muscle and liver of Atlantic salmon (*Salmo salar* L.). *In preparation*.

3.1 ABSTRACT

Vegetable oils (VO) are globally accepted alternatives for fish oil (FO) in aquafeeds. The lack of n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA) in VO is a major constraint for their use. *Echium* oil (EO), rich in stearidonic acid (SDA), has the potential to increase endogenous n-3 LC-PUFA biosynthesis. We tested whether feeding Atlantic salmon an EO-based diet in both freshwater and seawater would increase n-3 LC-PUFA levels by comparing the fatty acid (FA) profiles in liver and white muscle to fish fed FO and rapeseed oil (RO)-based diets. The gene expression of n-3 LC-PUFA biosynthetic enzymes was measured to support the underlying mechanism of n-3 LC-PUFA biosynthesis. After prolonged feeding with EO diet from freshwater to seawater phases, EO fish had higher n-3 LC-PUFA levels in both liver and white muscle compared to RO fish. However, FO fish had the highest n-3 LC-PUFA levels in examined tissues. $\Delta 6$ Desaturase gene expression in liver and white muscle was up-regulated in RO fish only, liver $\Delta 5$ desaturase gene expression was reduced in seawater and liver FA elongase gene expression was regulated by an interaction of dietary oil and environment. This study showed that feeding Atlantic salmon from parr to smolt using an SDA enriched diet increases n-3 LC-PUFA biosynthesis by bypassing the initial $\Delta 6$ desaturase enzyme through increased supply of the n-3 LC-PUFA precursor SDA. In addition, the down regulation of $\Delta 5$ desaturase gene expression in the liver of seawater fish may explain environmental differences in n-3 LC-PUFA biosynthesis.

3.2 INTRODUCTION

The salmonid aquaculture industry uses 56% of the world fish oil (FO) production for aquafeed production (Tacon and Metian, 2008). The rise in the price of FO during recent years has challenged the industry to evaluate sustainable substitutes for FO. Promising results for various FO substitutes of vegetable origin have been obtained with respect to fish growth, performance and health (Rosenlund et al., 2001; Tocher et al., 2003; Torstensen et al., 2005). However, the lack of n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA), particularly eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in vegetable oils (VO) remains the main constraint. It is well known that the fatty acid (FA) composition of fish reflects the FA profile of their diets, hence using diets with high inclusion levels of VO results in low n-3 LC-PUFA content in fish (Torstensen et al., 2000; Bell et al., 2001; Bransden et al., 2003). A large body of evidence exists about the health benefits of high n-3 LC-PUFA in human diets (Tapiero et al., 2002; Ruxton et al., 2005, 2007). Atlantic salmon is traditionally known to contain large amounts of n-3 LC-PUFA and is an excellent source of these beneficial FA for human consumers. It is widely acknowledged that alternative oils in aquafeeds should not compromise fish n-3 LC-PUFA requirements or the associated human health benefits (Gatlin et al., 2007). Consequently, novel oil sources such as *Echium* oil have attracted attention (Bell et al., 2006; Tocher et al., 2006; Miller et al., 2007, 2008a, 2008b; Diaz-Lopez et al., 2009), as they may address, via provision of the precursor PUFA stearidonic acid (SDA; 18:4n-3), the low levels of n-3 LC-PUFA found in fish fed on VO by capitalizing on the endogenous n-3 LC-PUFA biosynthetic capacity of fish (Miller et al., 2007, 2008a, 2008b). In a recent study using SDA-containing EO, this has been demonstrated for Atlantic salmon in freshwater and seawater, by

examining changes in FA composition and determining the accretion of n-3 LC-PUFA by a fatty acid mass balance approach at the whole organism level (Chapter 2 and Codabaccus et al., 2011).

All vertebrates including fish are capable of converting α -linolenic acid (ALA; 18:3n-3) to EPA and DHA along the n-3 biosynthetic pathway through a series of elongase and desaturases enzymes. The bioconversion of n-3 LC-PUFA is especially inefficient for marine fish species (Sargent et al., 2002; Tocher, 2003) and the first step along the pathway, the conversion of ALA to SDA by $\Delta 6$ desaturase, is a limiting step (Brenner, 1981). *Echium* oil (EO) contains ~14% SDA, which is the immediate product of the desaturation of ALA. Feeding fish with dietary EO will be reflected by high SDA content in fish; concomitantly its position along the n-3 biosynthetic pathway may cause the first $\Delta 6$ desaturase enzyme to be bypassed through product feedback inhibition and supply of extra substrate (SDA) for increased bioconversion to n-3 LC-PUFA. To verify this hypothesis, we compared the FA composition of the liver and white muscle of Atlantic salmon fed from freshwater to seawater a diet where FO was completely substituted by either EO or rapeseed oil (RO). The choice of rapeseed oil in this present study as a source of ALA was prompted by analogy to two independent studies with Atlantic salmon parr and smolt conducted in freshwater and seawater respectively; whereby rapeseed oil-based diet was used as control (Miller et al., 2007, 2008b). Elongase, $\Delta 6$ desaturase and $\Delta 5$ desaturase enzyme gene expression was also measured in these two tissues to support our hypothesis and to investigate any environmental regulation. In a previous study undertaken with Atlantic salmon (smolt) fed an EO diet for 84 days in seawater (Miller et al., 2008b); liver gene expression of $\Delta 5$ desaturase and elongase was up-

regulated which was reflected in increased EPA content in the liver. However total n-3 LC-PUFA content was not increased in the tissues examined (Miller et al., 2008b). Atlantic salmon being an anadromous fish has to migrate to seawater and as a result exhibits changes in n-3 LC-PUFA metabolism both prior to and after seawater migration (Bell et al., 1997; Tocher et al., 2000, 2003). Therefore a critical difference between the previous and the present study is that here, Atlantic salmon were fed with EO diet for a considerably longer period (196 days total feeding) that encompassed growing the fish through the transition from freshwater (parr) to seawater (smolt). To my knowledge this study is the first examining the in vivo gene expression in tissues of Atlantic salmon fed an SDA-rich diet from freshwater to seawater.

3.3 MATERIALS and METHODS

3.3.1 *Experimental diets*

Three diets were formulated to compare rapeseed oil (RO), *Echium* oil (EO) and fish oil (FO) (Table 3.1) and prepared as previously described (Chapter 2 and Codabaccus et al., 2011).

3.3.2 *Growth experiment*

The experiment was conducted at the University of Tasmania (Launceston, Tasmania, Australia) in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0009731). Fish housing, husbandry and feeding protocol has previously been described in detail (Chapter 2 and Codabaccus et al., 2011).

Atlantic salmon parr of average weight ~25 g were fed one of three diets; RO, EO or FO at an initial ration of 2.0% body weight per d (%BW/d). Every 14 days, feed intake was monitored to adjust feeding ration. Based on physical characteristics of smolting fish such as silvering of the body, loss of parr marks and darkening of the fin margins (McCormick et al., 2000), fish were transferred to seawater after being reared in freshwater for a period of 112 days. Prior to seawater transfer, fish were bulk weighed and six fish per treatment were euthanized (100 mg/L benzocaine). Muscle from below the dorsal fin and liver were dissected, and stored at -20°C until fatty acid analyses. Fish were grown for a further 84 days in seawater and muscle and liver of six fish per treatment were collected for fatty acid analyses.

3.3.3 Lipid extraction and analyses

Samples were freeze-dried and extracted overnight using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single-phase overnight extraction, CHCl₃: MeOH: H₂O (1:2:0.8, v:v:v), followed by phase separation to yield a total lipid extract. Lipid classes were analyzed by an Iatroscan MK V TLC-flame ionization detector analyzer (Iatron Laboratories). An aliquot of the total lipid extract was transmethylated in methanol:chloroform:hydrochloric acid (10:1:1, v:v:v) for 2 h at 100°C to obtain fatty acid methyl esters (FAME). GC was performed with an Agilent Technologies 7890B GC fitted with a Supelco Equity-1 fused silica capillary column (15 m x 0.1 mm i.d., 0.1 µm film thickness), a flame ionization detector, a split/splitless injector, and an Agilent Technologies 7683B Series autosampler. Helium was the carrier gas. Individual components were identified by mass spectral data and by comparing retention time data with authentic and laboratory standards. GC-mass spectrometric (GC-MS) analyses were performed to confirm component

identification on a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, Texas, USA).

Table 3.1: Ingredient and lipid composition (g/kg dry matter) of diets of Atlantic salmon fed EO, FO and RO

	Diet		
	EO	FO	RO
<i>Ingredient composition (g /kg)</i>			
Fishmeal (defatted) ¹	250	250	250
Casein ²	50	50	50
Wheat gluten ³	100	100	100
Soybean meal ⁴	189	189	189
Fish oil ¹	0	200	0
Rapeseed oil ⁵	0	0	200
Echium oil ⁶	200	0	0
Pre gel starch ⁷	127	127	127
Vitamin mix ⁸	3	3	3
Mineral mix ⁹	5	5	5
Stay C ¹⁰	3	3	3
Choline chloride ¹¹	2	2	2
Sipernat ¹²	40	40	40
CMC ¹¹	10	10	10
Monobasic calcium phosphate ¹¹	20	20	20
Yttrium oxide ¹¹	1	1	1
<i>Chemical composition</i>			
Dry matter	911.3	905.0	907.7
Crude protein	342.3	346.8	345.8
Total lipid	213.0	215.9	213.7
Energy (MJ/kg DM)	19.7	19.7	19.7
<i>g/ kg DM</i>			
Total SFA	24.7	52.9	20.9
Total MUFA	36.8	51.1	123.5
18:3n-3	41.0	2.0	14.4
18:4n-3	15.7	4.3	0.1
20:5n-3	0.3	28.3	0.9
22:6n-3	0.4	9.8	0.6
Totaln-3	57.4	50.3	16.1
18:2n-6	36.2	9.4	41.4
18:3n-6	14.6	0.6	0.1
Totaln-6	51.1	13.0	41.5
Total PUFA	108.5	67.5	57.7

¹Skretting Australia, Cambridge, Tasmania, Australia; ²MP Biomedicals Australasia Pty Ltd, Seven Hills NSW, Australia; ³Starch Australasia, Lane Cove, NSW, Australia; ⁴Hamlet Protein A/S, Horstens, Denmark; ⁵Croda Chemicals, East Yorkshire, UK; ⁶Steric Trading Pty Ltd, Villawood, NSW, Australia; ⁷Penford Limited, Lane Cove, NSW, Australia; ⁸Vitamin mix (ASV4) (Carter et al., 2003); ⁹Mineral mix (TMV4) (Carter et al., 2003); ¹⁰Roche Vitamins Australia, Frenchs Forest, NSW, Australia; ¹¹Sigma-Aldrich, Castle Hill, NSW, Australia; ¹²Degussa, Frankfurt, Germany, EO, *Echium* oil diet; RO, rapeseed oil diet; FO, fish oil diet, SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CMC, Carboxymethyl cellulose.

3.3.5 RNA isolation and preparation

Total RNA was extracted from muscle and liver tissue from freshwater and seawater samples ($n = 6$) stored in an RNA preservation reagent (25mmol/L sodium citrate, 10 mmol/L EDTA, 10 mol/L ammonium sulphate, pH 5.2) and purified using TRI Reagent (Molecular Research Center), including DNase treatment (DNA-free, Ambion). RNA yield (A260) and purity (A260/230 and A260/280) were determined spectrophotometrically and the integrity of the RNA was estimated from gel electrophoresis on a 1% agarose gel.

3.3.6 Reverse transcription

First-strand cDNA was synthesized from total RNA (0.5 μ g) using a SensiMix kit (Quantace) with oligo(dT)18 priming according to the manufacturer's instructions. The reactions were incubated at 65°C for 10 min, and then 42°C for 50 min before the RT enzyme was inactivated at 70°C for 15 min. First-strand cDNA reactions (20 μ L) were diluted to 80 μ L using nuclease-free water (Sigma-Aldrich) and stored at -80°C before quantitative PCR (qPCR).

3.3.7 qPCR

Real-time PCR primers were designed previously (Miller et al., 2008b) using gene sequences available on GenBank and a 1147-bp expressed sequence tag contig (SGP.Contig7470) identified as polyubiquitin (PolyUb) (94% nucleotide identity to *O. mykiss* PolyUb; accession no. AF361365) by searching the salmon genome project database. The RNA polymerase II primers were designed previously (Jorgensen et al., 2006) from a 556-bp salmon sequence (accession no. CA049789). qPCR used SYBR Green chemistry on a MyiQ Real-Time PCR Detection system (Bio-Rad). Each

reaction (10 μ L) contained primers (200 nmol/L each), SensiMixPlus SYBR and Fluorescein PCR master mix (Quantace), and 2 μ L cDNA. All samples were assayed for each gene in duplicate with no template controls and a 5-step, 3-fold cDNA dilution series for PCR efficiency calculation on the same plate. The reaction was incubated at 95°C for 10 min to activate the heat-activated DNA polymerase followed by 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 25 s. At the end of the 40 cycles, a melt curve analysis was performed to test the specificity of reaction.

3.3.8 *Relative expression*

mRNA expression levels were normalized using the geometric mean of 3 stably expressed reference genes (β -actin, RNA polymerase II, and PolyUb as determined by the geNorm software) (Vandesompele et al., 2002). Automated analysis of qPCR data used qBase software (Hellemans et al., 2007), with a modified $\Delta\Delta$ -Ct relative quantification model with PCR efficiency correction and multiple reference gene normalization.

3.3.9 *Statistical analysis*

Values ($n = 6$) are reported as means \pm SEM. Normality and homogeneity of variance were confirmed prior to analysis. Comparison between treatments of FA concentration and mean normalized relative quantities was by 2-way ANOVA followed by multiple comparisons using Tukey-Kramer honestly significant difference at $P < 0.05$. SPSS for Windows version 16 was used for statistical analysis.

3.4 RESULTS

3.4.1 *Lipid class composition and lipid content – liver and white muscle*

In the liver, there were dietary differences in free fatty acids (FFA) and polar lipids (PL), with higher FFA for RO fish and higher PL for FO fish. There was higher lipid content in the liver for fish in seawater compared to fish in freshwater (Table 3.2).

In the muscle, there were more sterols for EO fish. Freshwater fish had more triacylglycerol (TAG) than seawater fish and seawater fish had more PL than freshwater fish. Seawater fish had lower lipid content compared to fish in freshwater (Table 3.3).

3.4.2 Fatty acid composition (%) - liver

Higher monounsaturated fatty acids (MUFA) occurred in the liver of RO fish. There was higher ALA and SDA in EO fish liver in both freshwater and seawater (Table 3.2). Fish in freshwater had more ALA and SDA in the liver compared to fish in seawater. There were no significant differences in relative levels (% composition of total fatty acids) of n-3 LC-PUFA between treatments in freshwater while in seawater, n-3 LC-PUFA % composition was in the order FO > EO > RO. There was no significant difference in DHA between treatments in freshwater, and FO fish had the highest DHA % composition in seawater. There was no significant difference in EPA between FO and EO fish both in freshwater and seawater, while RO fish had lower EPA % composition in both freshwater and seawater compared to EO and FO fish. EO fish had the highest eicosatetraenoic acid (ETA; 20:4n-3) levels in both freshwater and particularly in seawater. Docosapentaenoic acid (DPA; 22:5n-3) levels of EO fish were higher than for RO fish in both freshwater and seawater. There was no significant difference in total n-3 between EO and FO fish both in freshwater and seawater, while RO fish had lower total n-3 (Table 3.2). There was no significant

difference in linoleic acid (LA; 18:2n-6) between EO and RO fish in both freshwater and seawater. EO fish had the highest γ -linolenic acid (GLA; 18:3n-6) and 20:3n-6 in freshwater and seawater compared to FO and RO fish (Table 3.2). Fish in freshwater had more GLA than fish in seawater. There was a significant interaction (diet x salinity) for arachidonic acid (ARA; 20:4n-6) with RO fish in seawater having highest ARA. Total n-6 PUFA was higher for EO and RO fish in both freshwater and seawater compared to FO fish. EO fish had highest total PUFA in freshwater and in seawater the total PUFA in EO and FO fish was not significantly different. The n-3: n-6 ratio was in the order FO > EO > RO.

3.4.3 Fatty acid composition (%) - white muscle

The FA composition of white muscle mirrored the FA composition of respective diets in both freshwater and seawater, with highest MUFA in RO fish, highest ALA and SDA in EO fish and highest n-3 LC-PUFA in FO fish (Table 3.3). Significant differences in n-3 LC-PUFA existed between RO and EO fish. EO fish had higher n-3 LC-PUFA than RO fish in both freshwater and seawater as a result of higher ETA, EPA, DPA and DHA (Table 3.3). EO fish had highest total n-3 and total PUFA compared to RO and FO fish. Significant interaction (diet x salinity) occurred for LA % composition with the highest levels for RO fish in seawater (Table 3.3). EO fish had the highest GLA in freshwater and seawater compared to FO and RO fish. There was significant interaction (diet x salinity) for 20:3n-6 with EO fish in seawater having highest levels. FO fish had the highest ARA in freshwater and seawater, with ARA lower in freshwater fish compared to seawater fish. Total n-6 PUFA was highest for EO fish in freshwater and seawater. The n-3: n-6 ratio was in the order FO > EO > RO.

Table 3.2: Fatty acid composition (as % of total fatty acids), lipid class composition and lipid content in the liver of Atlantic salmon fed EO, FO and RO diets (mean \pm SEM)

FA (%)	Freshwater						Seawater					
	EO		FO		RO		EO		FO		RO	
Total SFA ¹	33.2	\pm 0.7 ^y	46.4	\pm 1.5 ^x	24.0	\pm 1.8 ^z	34.8	\pm 2.3 ^y	33.9	\pm 1.5 ^y	22.5	\pm 1.5 ^z
Total MUFA ²	24.2	\pm 0.9 ^c	29.8	\pm 0.9 ^b	45.7	\pm 1.2 ^a	23.7	\pm 2.2 ^C	31.5	\pm 3.3 ^B	48.8	\pm 1.6 ^A
18:3n-3	4.4	\pm 0.2 ^a	0.6	\pm 0.1 ^b	1.4	\pm 0.2 ^b	3.7	\pm 0.3 ^{A*}	0.3	\pm 0.1 ^{B*}	0.5	\pm 0.2 ^{B*}
18:4n-3	3.6	\pm 0.3 ^a	0.5	\pm 0.2 ^b	1.2	\pm 0.1 ^b	3.4	\pm 0.5 ^{A*}	0.3	\pm 0.1 ^{B*}	0.4	\pm 0.1 ^{B*}
20:4n-3	1.2	\pm 0.1 ^a	0.2	\pm 0.1 ^b	0.2	\pm 0.1 ^b	1.2	\pm 0.3 ^A	0.3	\pm 0.1 ^B	0.1	\pm 0.1 ^B
20:5n-3	4.9	\pm 0.3 ^a	5.3	\pm 0.6 ^a	2.7	\pm 0.2 ^b	4.9	\pm 0.7 ^A	5.7	\pm 0.8 ^A	1.6	\pm 0.1 ^B
22:5n-3	1.4	\pm 0.1 ^{ab}	1.7	\pm 0.2 ^a	1.2	\pm 0.1 ^b	1.7	\pm 0.4 ^{AB}	2.5	\pm 0.5 ^A	0.9	\pm 0.1 ^B
22:6n-3	8.2	\pm 0.7 ^y	8.6	\pm 1.0 ^y	6.4	\pm 0.4 ^y	9.1	\pm 1.8 ^y	16.7	\pm 1.6 ^x	5.4	\pm 0.5 ^y
n-3 LC-PUFA	15.7	\pm 1.2 ^{yz}	15.9	\pm 1.8 ^{yz}	10.5	\pm 0.1 ^{yz}	16.9	\pm 3.1 ^y	25.3	\pm 2.8 ^x	8.0	\pm 0.7 ^z
Total n-3 ³	23.8	\pm 1.2 ^x	17.1	\pm 1.7 ^{xy}	13.3	\pm 0.7 ^y	23.9	\pm 3.7 ^x	26.1	\pm 3.0 ^x	9.0	\pm 0.9 ^y
18:2n-6	9.5	\pm 0.3 ^a	2.9	\pm 0.2 ^b	9.4	\pm 0.5 ^a	8.6	\pm 0.2 ^A	4.1	\pm 1.3 ^B	9.2	\pm 0.4 ^A
18:3n-6	2.1	\pm 0.1 ^a	0.2	\pm 0.1 ^c	1.2	\pm 0.1 ^b	1.6	\pm 0.1 ^{A*}	0.1	\pm 0.1 ^{C*}	1.1	\pm 0.1 ^{B*}
20:3n-6	3.6	\pm 0.4 ^a	0.2	\pm 0.1 ^c	2.4	\pm 0.2 ^b	3.5	\pm 0.3 ^A	0.4	\pm 0.1 ^C	3.1	\pm 0.2 ^B
20:4n-6	2.7	\pm 0.2 ^y	2.4	\pm 0.3 ^y	2.6	\pm 0.2 ^y	3.5	\pm 0.4 ^y	3.0	\pm 0.2 ^y	5.0	\pm 0.4 ^x
Total n-6 ⁴	18.8	\pm 0.9 ^a	6.5	\pm 0.5 ^b	17.0	\pm 0.4 ^a	17.6	\pm 0.7 ^A	8.3	\pm 1.3 ^B	19.7	\pm 0.6 ^A
Total PUFA ⁵	42.6	\pm 1.1 ^x	23.9	\pm 2.1 ^y	30.3	\pm 1.0 ^y	41.5	\pm 4.4 ^x	34.4	\pm 3.0 ^{xy}	28.7	\pm 1.4 ^y
n-3 : n-6	1.3	\pm 0.1 ^b	2.6	\pm 0.2 ^a	0.8	\pm 0.0 ^c	1.3	\pm 0.2 ^B	3.5	\pm 0.6 ^A	0.5	\pm 0.0 ^C
Lipid Class (%)												
HC	3.5	\pm 0.9	1.3	\pm 0.3	3.0	\pm 0.4	2.3	\pm 0.3	1.9	\pm 0.6	2.3	\pm 0.8
TAG	0.6	\pm 0.3	3.0	\pm 1.4	0.6	\pm 0.2	0.1	\pm 0.1	0.4	\pm 0.3	0.9	\pm 0.4
FFA	10.2	\pm 0.6 ^b	5.4	\pm 0.6 ^c	12.0	\pm 1.3 ^a	7.4	\pm 1.0 ^B	6.7	\pm 0.9 ^C	11.4	\pm 1.1 ^A
ST	4.4	\pm 0.8	5.6	\pm 0.8	4.1	\pm 0.7	6.6	\pm 0.8 [*]	5.5	\pm 0.5 [*]	5.5	\pm 0.6 [*]
PL	81.3	\pm 0.8 ^{ab}	84.7	\pm 1.1 ^a	80.4	\pm 1.2 ^b	83.6	\pm 1.8 ^{AB}	85.5	\pm 0.8 ^A	79.9	\pm 1.9 ^B
Lipid content (mg/g) ⁶												
Wet	24.7	\pm 1.4	21.2	\pm 0.9	26.4	\pm 2.2	29.0	\pm 2.7 [*]	28.4	\pm 1.5 [*]	33.6	\pm 3.5 [*]
Dry	95.9	\pm 6.1	88.8	\pm 5.0	106.6	\pm 8.5	122.0	\pm 11.2 [*]	118.5	\pm 5.6 [*]	137.1	\pm 12.7 [*]

^{a,b,c,A,B,C} Mean values across a row not sharing a common superscript represents dietary differences, similar small and capital letters between freshwater and seawater were not significantly different. $P < 0.05$; ^{x,y,z} Mean values across a row not sharing a common superscript represents interaction (diet x salinity) differences. $P < 0.05$; An * represents significant differences between freshwater and seawater treatments, $P < 0.05$; HC, hydrocarbon; TAG, triacylglycerol; FFA, free fatty acid; ST, sterol; PL, polar lipid; ¹ Includes 14:0, 15:0, 17:0, 18:0, 20:0, 22:0 and 24:0; ² Includes 16:1n-7, 16:1n-9, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-7, 20:n-9, 22:1n-9, 22:1n-11 and 24:1n-9; ³ Includes 21:5n-3 and 24:6n-3; ⁴ Includes 20:2n-6, 22:4n-6 and 24:5n-6; ⁵ Includes 16:2n-4, 16:3n-4 and 18:2n-9; ⁶ Determined gravimetrically; EO, *Echium* oil diet; RO, rapeseed oil diet; FO, fish oil diet.

Table 3.3: Fatty acid composition (as % total fatty acids), lipid class composition and lipid content in the muscle of Atlantic salmon fed EO, FO and RO diets (mean \pm SEM)

FA (%)	Freshwater						Seawater					
	EO		FO		RO		EO		FO		RO	
Total SFA ¹	21.0	\pm 0.3 ^b	30.9	\pm 0.9 ^a	15.7	\pm 0.3 ^c	22.1	\pm 0.6 ^B	31.5	\pm 0.8 ^A	15.6	\pm 0.6 ^C
Total MUFA ²	25.5	\pm 0.4 ^c	32.9	\pm 0.9 ^b	55.4	\pm 0.3 ^a	22.7	\pm 1.1 ^C	32.7	\pm 2.0 ^B	54.0	\pm 1.2 ^A
18:3n-3	12.4	\pm 0.3 ^a	1.0	\pm 0.1 ^c	3.4	\pm 0.1 ^b	12.4	\pm 0.1 ^A	1.0	\pm 0.1 ^C	3.2	\pm 0.1 ^B
18:4n-3	8.2	\pm 0.2 ^a	2.1	\pm 0.1 ^b	1.6	\pm 0.0 ^c	8.0	\pm 0.3 ^A	1.8	\pm 0.1 ^B	1.6	\pm 0.0 ^C
20:4n-3	0.7	\pm 0.3 ^a	0.4	\pm 0.1 ^b	0.1	\pm 0.0 ^b	1.5	\pm 0.1 ^{A*}	0.7	\pm 0.1 ^{B*}	0.4	\pm 0.0 ^{B*}
20:5n-3	2.9	\pm 0.2 ^b	9.7	\pm 0.2 ^a	1.9	\pm 0.1 ^c	3.2	\pm 0.4 ^B	9.5	\pm 0.3 ^A	1.5	\pm 0.1 ^C
22:5n-3	1.3	\pm 0.1 ^b	3.7	\pm 0.1 ^a	0.8	\pm 0.1 ^c	1.3	\pm 0.1 ^B	3.8	\pm 0.2 ^A	0.7	\pm 0.0 ^C
22:6n-3	5.2	\pm 0.5 ^b	10.9	\pm 0.8 ^a	3.7	\pm 0.2 ^c	6.1	\pm 1.0 ^B	11.3	\pm 1.1 ^A	3.4	\pm 0.4 ^C
n-3 LC-PUFA	10.2	\pm 0.7 ^b	24.7	\pm 0.9 ^a	6.5	\pm 0.4 ^c	12.2	\pm 1.5 ^B	25.4	\pm 1.4 ^A	6.0	\pm 0.5 ^C
Total n-3 ³	30.9	\pm 0.6 ^a	28.4	\pm 0.8 ^b	11.6	\pm 0.3 ^c	32.7	\pm 1.2 ^A	28.6	\pm 1.4 ^B	10.7	\pm 0.6 ^C
18:2n-6	14.5	\pm 0.2 ^{xy}	4.4	\pm 0.1 ^z	13.5	\pm 0.2 ^y	14.5	\pm 0.5 ^{xy}	4.4	\pm 0.2 ^z	15.0	\pm 0.3 ^x
18:3n-6	4.9	\pm 0.1 ^a	0.3	\pm 0.0 ^c	1.2	\pm 0.0 ^b	4.7	\pm 0.2 ^A	0.2	\pm 0.1 ^C	1.4	\pm 0.0 ^B
20:3n-6	1.8	\pm 0.0 ^y	0.3	\pm 0.0 ^z	1.1	\pm 0.0 ^y	2.2	\pm 0.1 ^x	0.2	\pm 0.1 ^z	1.5	\pm 0.1 ^y
20:4n-6	0.6	\pm 0.1 ^b	0.9	\pm 0.0 ^a	0.6	\pm 0.0 ^b	0.8	\pm 0.1 ^{B*}	1.0	\pm 0.0 ^{A*}	0.9	\pm 0.1 ^{B*}
Total n-6 ⁴	22.4	\pm 0.3 ^w	6.6	\pm 0.1 ^z	17.3	\pm 0.2 ^y	22.5	\pm 0.5 ^w	6.3	\pm 0.2 ^z	19.6	\pm 0.4 ^x
Total PUFA ⁵	53.5	\pm 0.6 ^a	36.1	\pm 0.8 ^b	28.9	\pm 0.2 ^c	55.2	\pm 0.9 ^A	35.7	\pm 1.3 ^B	30.4	\pm 0.8 ^C
n-3 : n-6	1.4	\pm 0.0 ^b	4.3	\pm 0.2 ^a	0.7	\pm 0.0 ^c	1.5	\pm 0.1 ^B	4.6	\pm 0.3 ^A	0.5	\pm 0.0 ^C
Lipid Class (%)												
TAG	71.1	\pm 3.1	77.9	\pm 4.5	83.0	\pm 2.4	59.8	\pm 10.4 [*]	69.8	\pm 5.3 [*]	68.3	\pm 6.8 [*]
FFA	3.6	\pm 0.6	2.3	\pm 0.3	2.4	\pm 0.1	2.8	\pm 0.7	2.1	\pm 0.5	2.2	\pm 0.4
ST	2.6	\pm 0.4 ^a	1.2	\pm 0.3 ^b	1.6	\pm 0.1 ^{ab}	2.7	\pm 0.8 ^A	1.4	\pm 0.4 ^B	1.8	\pm 0.4 ^{AB}
PL	22.7	\pm 2.8	18.5	\pm 4.2	13.0	\pm 2.3	34.7	\pm 9.0 [*]	26.9	\pm 4.6 [*]	27.6	\pm 6.3 [*]
Lipid content (mg/g) ⁶												
Wet	26.0	\pm 2.7	21.0	\pm 3.7	26.7	\pm 1.0	16.4	\pm 3.0 [*]	19.0	\pm 3.4 [*]	18.0	\pm 3.3 [*]
Dry	102.8	\pm 8.9	81.6	\pm 13.5	103.2	\pm 4.4	63.7	\pm 9.9 [*]	73.3	\pm 13.0 [*]	72.9	\pm 12.9 [*]

a,b,c,A,B,C Mean values across a row not sharing a common superscript represents dietary differences, similar small and capital letters between freshwater and seawater were not significantly different. $P < 0.05$; ^{x,y,z} Mean values across a row not sharing a common superscript represents interaction (diet x salinity) differences. $P < 0.05$; An * represents significant differences between freshwater and seawater treatments, $P < 0.05$; HC, hydrocarbon; TAG, triacylglycerol; FFA, free fatty acid; ST, sterol; PL, polar lipid; ¹ Includes 14:0, 15:0, 17:0, 18:0, 20:0, 22:0 and 24:0; ² Includes 16:1n-7, 16:1n-9, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-7, 20:n-9, 22:1n-9, 22:1n-11 and 24:1n-9; ³ Includes 21:5n-3 and 24:6n-3; ⁴ Includes 20:2n-6, 22:4n-6 and 24:5n-6; ⁵ Includes 16:2n-4, 16:3n-4 and 18:2n-9; ⁶ Determined gravimetrically; EO, *Echium* oil diet; RO, rapeseed oil diet; FO, fish oil diet.

Table 3.4: Summary of two way ANOVA of fatty acid and lipid class composition in the liver and muscle of Atlantic salmon fed *Echium* oil (EO) fish oil (FO) and rapeseed oil (RO) diets

FA	Liver						Muscle					
	Diet		Salinity		Interaction		Diet		Salinity		Interaction	
	F-value	P- value	F-value	P- value	F-value	P- value	F-value	P- value	F-value	P- value	F-value	P- value
Total SFA	55.3	< 0.01	9.7	< 0.01	10.3	< 0.01	326.6	< 0.01	1.2	0.29	0.5	0.64
Total MUFA	81.7	< 0.01	0.9	0.36	0.5	0.63	398.2	< 0.01	2.6	0.12	0.7	0.53
18:3n-3	212.0	< 0.01	16.0	< 0.01	1.4	0.26	3390.8	< 0.01	0.2	0.69	0.3	0.76
18:4n-3	87.3	< 0.01	4.3	0.05	0.9	0.41	1445.4	< 0.01	2.5	0.12	0.7	0.52
20:4n-3	15.3	< 0.01	0.0	0.84	0.1	0.92	19.7	< 0.01	16.1	< 0.01	2.4	0.11
20:5n-3	25.8	< 0.01	0.4	0.53	1.4	0.27	659.3	< 0.01	0.2	0.64	1.1	0.35
22:5n-3	7.0	< 0.01	1.2	0.28	2.0	0.15	446.1	< 0.01	0.0	0.94	0.8	0.47
22:6n-3	18.8	< 0.01	8.5	0.01	9.2	0.00	56.8	< 0.01	0.3	0.58	0.3	0.74
n-3 LC-PUFA	17.4	< 0.01	2.9	0.10	5.0	< 0.01	191.3	< 0.01	0.8	0.39	0.8	0.45
Total n-3	19.5	< 0.01	0.8	0.38	4.8	< 0.05	307.2	< 0.01	0.2	0.63	1.0	0.37
18:2n-6	53.4	< 0.01	0.0	0.97	1.4	0.26	929.5	< 0.01	4.3	< 0.05	5.2	< 0.05
18:3n-6	114.6	< 0.01	8.2	< 0.01	2.4	0.11	907.7	< 0.01	0.0	0.87	2.2	0.12
20:3n-6	96.1	< 0.01	1.5	0.23	1.7	0.20	264.6	< 0.01	17.9	< 0.01	6.8	< 0.01
20:4n-6	7.7	< 0.01	27.8	< 0.01	5.8	< 0.01	7.5	< 0.01	17.5	< 0.01	3.0	0.07
Total n-6	125.0	< 0.01	2.9	0.10	3.2	0.05	1361.4	< 0.01	7.4	< 0.05	10.0	< 0.01
Total PUFA	17.5	< 0.01	1.7	0.21	3.8	< 0.05	488.5	< 0.01	1.7	0.20	1.0	0.39
n-3 : n-6	46.6	< 0.01	0.7	0.40	2.7	0.09	348.5	< 0.01	0.4	0.56	0.8	0.45
Lipid Class (%)												
HC	2.8	0.08	1.0	0.32	1.3	0.29	na	na	na	Na	na	na
TAG	2.7	0.08	3.1	0.09	3.0	0.06	2.8	0.21	5.3	< 0.05	0.1	0.86
FFA	17.7	< 0.01	0.8	0.37	2.2	0.13	2.8	0.08	1.3	0.27	0.3	0.73
ST	0.8	0.48	4.3	< 0.05	1.3	0.28	4.8	< 0.05	0.1	0.75	0.0	0.99
PL	6.8	< 0.01	0.6	0.44	0.5	0.62	1.3	0.29	7.1	< 0.05	0.2	0.84
Lipid content (mg/g)												
Wet	2.8	0.08	11.9	< 0.01	0.3	0.75	0.3	0.73	7.8	< 0.01	1.0	0.38
Dry	2.3	0.11	16.5	< 0.01	0.0	0.96	0.5	0.63	8.4	< 0.01	1.1	0.36

3.4.4 EPA, DHA and n-3 LC-PUFA content (mg/g) - white muscle

EPA, DHA and n-3 LC-PUFA were significantly higher in the white muscle of FO fish (Fig 3.1). EPA and n-3 LC-PUFA were significantly higher in the white muscle EO fish compared to RO fish. There was no difference in DHA content in the white muscle between EO and RO fish. There was no significant difference in EPA, DHA and n-3 LC-PUFA content (mg/g) between freshwater and seawater fish, although values for freshwater fish were higher than seawater fish (EPA, 4.3 v/s 3.3; DHA, 5.9 v/s 4.6; n-3 LC-PUFA, 12.4 v/s 9.9).

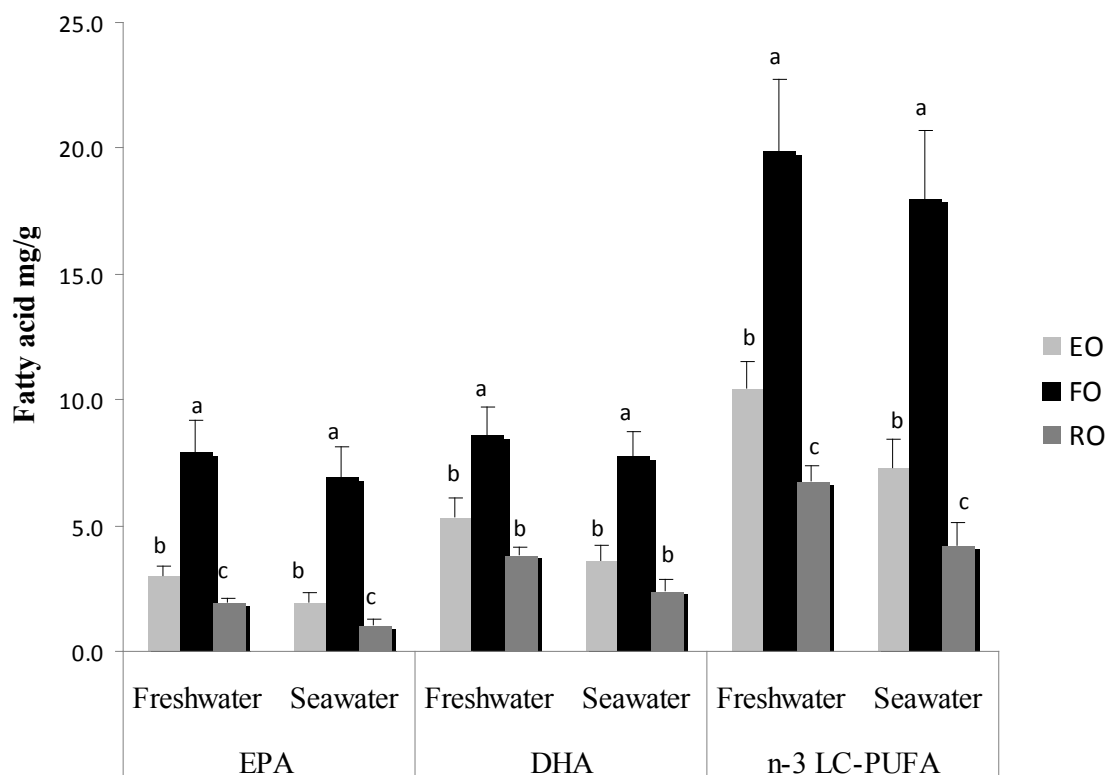
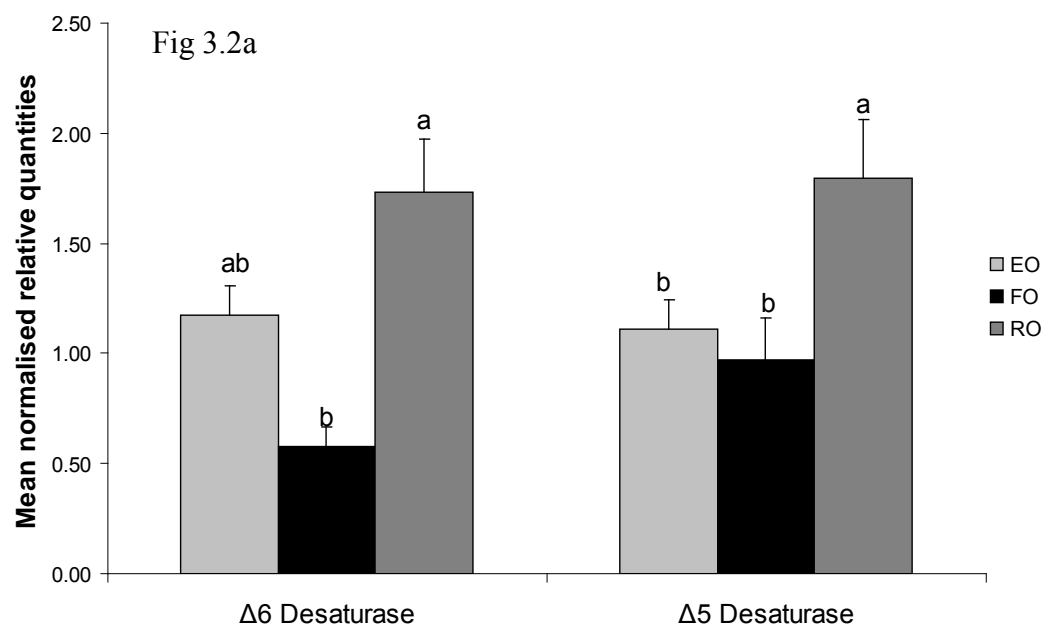


Figure 3.1: EPA, DHA and n-3 LC-PUFA content (mg/g, dry weight) in the white muscle of Atlantic salmon fed EO, RO and FO diets in freshwater and seawater. Values are mean \pm SEM, $n = 6$. Different letters represent significant dietary differences, $P < 0.05$.

3.4.5 Gene expression - liver

Measurement of mRNA abundance by qRT-PCR showed that gene expression in the liver was significantly affected by dietary oil (Fig 3.2). $\Delta 6$ desaturase gene expression in the

liver was upregulated in freshwater and seawater for RO fish compared to FO fish (Fig 3.2a). $\Delta 6$ desaturase gene expression in the liver for EO fish was intermediate between RO and FO fish. $\Delta 5$ desaturase gene expression was only upregulated in RO fish, both in freshwater and seawater. There was higher $\Delta 5$ desaturase gene expression between fish in freshwater compared to those in seawater (Fig 3.2b). There was significant interaction (diet x salinity) for elongase gene expression with only a clear difference between low expression in freshwater for EO and FO fish and high expression in seawater for EO fish (Fig 3.2c).



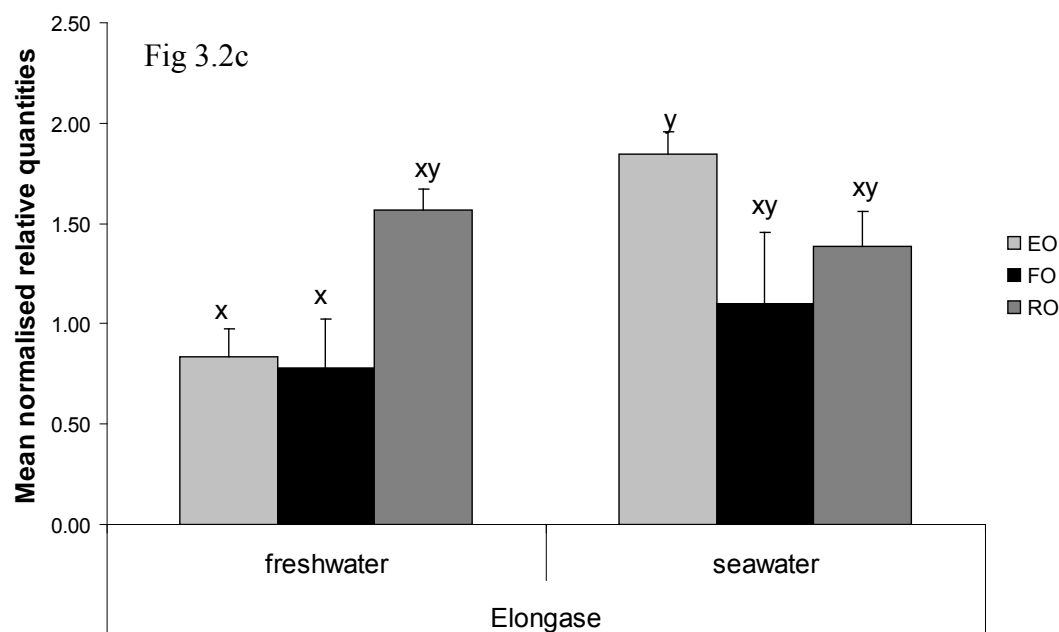
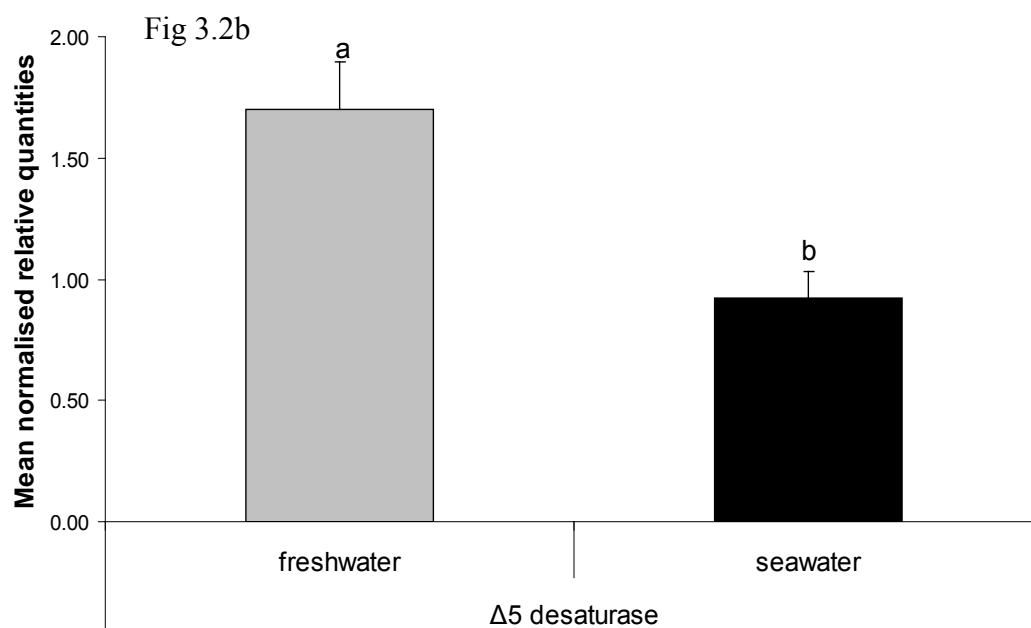


Figure 3.2: Differential gene expression of n-3 LC-PUFA biosynthetic enzymes in the liver of Atlantic salmon fed EO, FO and RO diets in freshwater and seawater. Values are mean \pm SEM, $n = 6$. **3.2a)** $\Delta 6$ and $\Delta 5$ Desaturase. Different letters represents significant dietary differences for $\Delta 6$ and $\Delta 5$ desaturase, $P < 0.05$. **3.2b)** $\Delta 5$ Desaturase. Different letters represent significant salinity differences for $\Delta 5$ desaturase, $P < 0.05$. **3.2c)** Elongase. Different letters represents significant interaction (diet x salinity) differences for elongase, $P < 0.05$.

3.4.6 Gene expression - white muscle

In the white muscle there were no significant dietary differences for $\Delta 5$ desaturase and elongase gene expression in either freshwater or seawater (Fig 3.3). $\Delta 6$ Desaturase gene expression was significantly upregulated in freshwater and seawater for RO fish compared to FO fish.

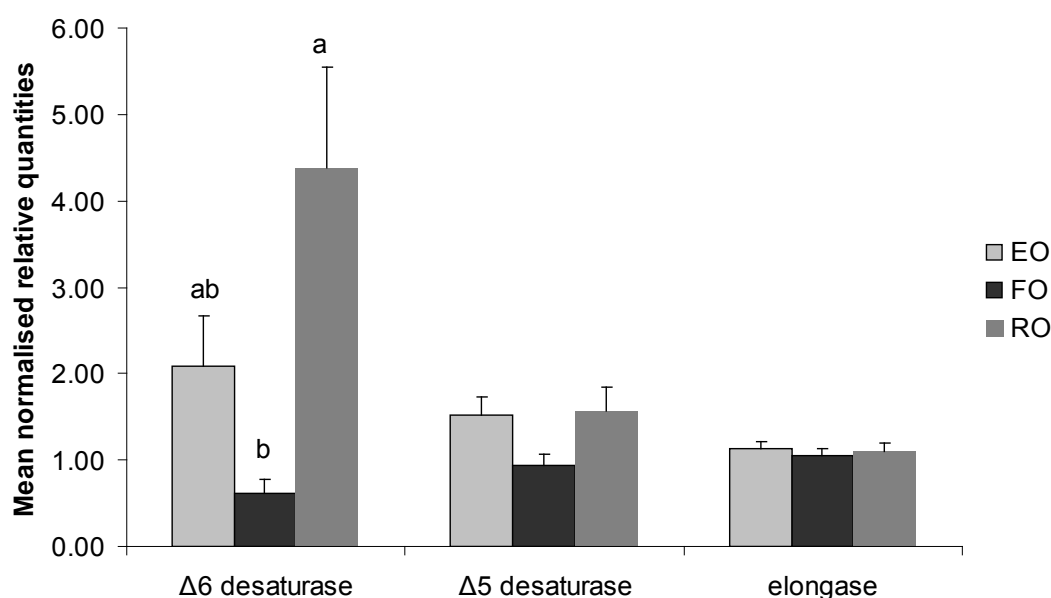


Figure 3.3: Differential gene expression of $\Delta 6$, $\Delta 5$ desaturase and elongase in the white muscle of Atlantic salmon fed EO, FO and RO diets. Values are mean \pm SEM, $n = 6$. Different letters represent significant dietary differences, $P < 0.05$.

Table 3.5: Summary of two way ANOVA on gene expression of $\Delta 6$ and $\Delta 5$ desaturases and elongase in the liver and muscle of Atlantic salmon fed EO, FO and RO diets in freshwater and seawater

Gene	Liver						Muscle					
	Diet		Salinity		Interaction		Diet		Salinity		Interaction	
	<i>P</i>	<i>f</i>	<i>P</i>	<i>f</i>	<i>P</i>	<i>f</i>	<i>P</i>	<i>f</i>	<i>P</i>	<i>f</i>	<i>P</i>	<i>f</i>
$\Delta 6$ desaturase	<0.05	13.1	0.33	1.0	0.60	0.6	<0.05	6.7	0.32	1.0	0.75	0.3
$\Delta 5$ desaturase	<0.05	14.5	<0.05	6.0	0.07	2.9	0.06	3.1	0.13	2.5	0.40	0.9
Elongase	0.06	3.2	<0.05	4.8	<0.05	4.2	0.78	0.3	0.76	0.1	0.97	0.0

3.5 DISCUSSION

3.5.1 *n-3 LC-PUFA composition and gene expression - liver*

The diets contained defatted fishmeal which resulted in only residual amounts of n-3 LC-PUFA in EO and RO feeds (Table 3.1). As a consequence, any differences in n-3 LC-PUFA biosynthesis between EO and RO fish should be reflected in the tissues examined. In freshwater as well as in seawater, ETA and EPA % composition in the liver of EO fish was higher than for RO fish. However, in freshwater, total n-3 LC-PUFA % composition was not significantly different between dietary treatments, although higher values were obtained in FO and EO fish. After an extended feeding period on EO, n-3 LC-PUFA % composition in the liver of EO fish in seawater was higher than that of RO fish. This difference reflects the trend of decreasing % composition of ETA, EPA, DPA and DHA in RO fish from freshwater to seawater. In this present study, EO fish continued to biosynthesize n-3 LC-PUFA at the same rate as in freshwater. Consequently, the % EPA in the liver was not different to FO fish. However, n-3 LC-PUFA in the liver of FO fish was higher than in EO fish in seawater most probably as a result of dietary n-3 LC-PUFA deposition particularly for DHA rather than biosynthesis.

The sustained % composition of n-3 LC-PUFA in the liver of EO and RO fish in freshwater and seawater is somewhat unexpected. In isolated hepatocytes of Atlantic salmon, desaturation of ALA to n-3 LC-PUFA was lower for Atlantic salmon in seawater compared to fish in freshwater prior to seawater transfer, therefore emphasizing the importance of environmental/genetic factors in the regulation of n-3 LC-PUFA biosynthetic activity (Bell et al., 1997; Tocher et al., 2000, 2003). The gene expression results in this present study showed lower expression of $\Delta 5$ desaturase in the seawater environment, suggesting the possible mechanism for a decrease in n-3 LC-PUFA biosynthesis. While gene expression is measured

at a fixed time point, the % composition of n-3 LC-PUFA is the result of lipid metabolism over a period of time. In a study on Atlantic salmon over the whole production cycle, the rate of conversion of ALA to n-3 LC-PUFA in hepatocytes was still high a few months after seawater transfer and the decrease in n-3 LC-PUFA biosynthetic activity in the marine environment was gradual for fish fed VO (Tocher et al., 2003). Therefore any difference in n-3 LC-PUFA between freshwater and seawater fish may only become apparent through a longer growth period. The gene expression of $\Delta 6$ and $\Delta 5$ desaturases is under nutritional regulation, amongst other factors (Jump et al., 1996, 1999; Zheng et al., 2005b). VO diets are known to increase the expression of those genes due to an abundance of dietary substrates such as ALA and LA with n-3 or n-6 LC-PUFA absent (Miller et al., 2008b). Interestingly, the $\Delta 5$ desaturase gene expression was not up-regulated in the liver of EO fish as opposed to RO fish. A bundance of substrate influences enzyme activity, but equally important is the removal or absence of the product. ETA, which is the substrate for $\Delta 5$ desaturase, was abundant in EO fish as was the product EPA. T his suggests that $\Delta 5$ desaturase gene expression was not up-regulated in EO fish due to sufficient enzymatic activity to reach the observed % EPA in the liver. In contrast, RO fish had low ETA in the liver, with $\Delta 5$ desaturase gene expression still up-regulated, indicating that up-regulation of desaturase gene expression is not just due to availability of substrate and/or product removal, but may also involve the physiological needs of fish for n-3 LC-PUFA. In a study conducted on smolt in seawater only, the gene expression of $\Delta 5$ desaturase in the liver of EO fish was up-regulated although the level of EPA was lower than that of FO fish (Miller et al., 2008b). Therefore the fish increased $\Delta 5$ desaturase enzyme activity to fulfill their requirements for n-3 LC-PUFA as opposed to observations in this present study. This difference was most probably due to the long term feeding on EO for fish in our study from parr to smolt.

Biosynthesis of n-3 LC-PUFA should not be assessed independently from n-6 LC-PUFA biosynthesis because the same desaturases and elongase act along both pathways (Tocher, 2003). In the present study, $\Delta 6$ desaturase gene expression in the liver was significantly up-regulated by dietary RO only. This supports our hypothesis that high dietary SDA may cause the $\Delta 6$ desaturase enzyme to be bypassed through product feedback inhibition. Theoretically, the $\Delta 6$ desaturase acts at three major steps along the PUFA biosynthetic pathways; from ALA to SDA, from LA to GLA and for the synthesis of DHA via the Sprecher pathway (Sprecher, 2000). It can also be suggested that high GLA from EO diet inhibited the $\Delta 6$ desaturase enzyme for EO fish. However, this scenario is unlikely; functional characterisation of Atlantic salmon $\Delta 6$ desaturase cDNA in the yeast (*Saccharomyces cerevisiae*) indicated a net preference towards the n-3 pathway when both n-3 and n-6 substrates were present (Zheng et al., 2005a; Leaver et al., 2008). Though we do not disregard the fact that some SDA could have been produced in EO fish, it is likely that the observed $\Delta 6$ desaturase gene expression for EO fish resulted in DHA biosynthesis. $\Delta 6$ Desaturase gene expression was up-regulated in RO fish and the presence of endogenous SDA, DHA and GLA indicates that $\Delta 6$ desaturase acted at both the n-3 and n-6 pathways. Enzyme activity towards a particular pathway may also depend on the relative concentration of substrates in the diet, albeit the net preference of $\Delta 6$ desaturase for the n-3 pathway. In RO diet, the ratio of LA: ALA was far higher than in the EO diet, hence enabling substantial $\Delta 6$ desaturase activity along the n-6 pathway.

Elongase gene expression is also under nutritional regulation and is positively correlated to dietary ALA (Leaver et al., 2008). However, interpreting elongase gene expression is more complex since it potentially acts at six steps along both the n-3 and n-6 biosynthetic pathways and has broad substrate specificity for PUFA with a range of chain

lengths from C18 to C22 (Zheng et al., 2004). In this present study, elongase gene expression was influenced by the combination of diet and environment which generally supports the view that expression may vary according to diet, environment and life cycle (Zheng et al., 2004, 2005b). The RO and EO diets up-regulated elongase gene expression in freshwater and seawater, respectively. In a previous study, the elongase gene expression in the liver of Atlantic salmon fed RO and EO diet was up-regulated and there was a preference for the n-6 pathway for elongase activity with high amounts of 20:3n-6 observed in tissues (Miller et al., 2008b). Similarly, in our study, the liver showed accumulation of 20:3n-6, the product of GLA elongation, in RO and EO fish both in freshwater and seawater, suggesting a preference towards the n-6 pathway. Very recently, a second elongase gene has been cloned for Atlantic salmon which is related to another elongase enzyme acting along the PUFA biosynthetic pathways (Tocher et al., 2010). Functional expression in yeast showed that it was predominantly active towards the elongation of C20 and C22 PUFA along both the n-3 and n-6 pathways (Tocher et al., 2010). The cloning of this new elongase gene will be useful to minimize the complexity surrounding elongase activity/gene expression studies along the n-3 and n-6 pathways for Atlantic salmon.

3.5.2 n-3 LC-PUFA composition and gene expression - white muscle

The FA composition and enzyme gene expression in the white muscle was quite different to the liver. The liver is the main organ for lipid metabolism and high n-3 LC-PUFA biosynthetic activity occurred as opposed to the white muscle which serves as a store of FA mainly as TAG. The FA composition in white muscle generally reflected the FA profile of their respective diets. $\Delta 5$ Desaturase and elongase genes were not overly expressed in the white muscle of VO fish compared to FO fish; this result is in agreement with a previous study (Zheng et al., 2005b).

EO fish had higher % n-3 LC-PUFA compared to RO fish in both freshwater and seawater, most probably due to the combined effects of bypassing the first $\Delta 6$ desaturase through supply of extra SDA substrate and long nutritional history of SDA rich dietary EO. The gene expression of $\Delta 6$ desaturase was upregulated for RO fish only and showed a similar pattern as in the liver. A previous study examined $\Delta 6$ desaturase gene expression in white muscle of Atlantic salmon smolt and showed similar findings (Miller, 2007). Consistent with the FA profiles in liver, there were no significant differences between % n-3 LC-PUFA in freshwater and seawater within dietary treatments for RO and EO fish in the white muscle. The presumption is that any differences may become apparent over longer growth periods as mentioned earlier. The growth and n-3 LC-PUFA metabolism of fish in this present experiment have also been examined at the whole organism level (Codabaccus et al., 2011). Growth was not different between EO and FO fish, and n-3 LC-PUFA biosynthesis was negligible in seawater at the whole body level. Low n-3 LC-PUFA biosynthetic activity and β -oxidation of substantial amounts of FA in seawater was identified as reasons for the observed difference between n-3 LC-PUFA biosynthesis in freshwater and seawater. In summary, at the whole body level an SDA rich diet enhanced n-3 LC-PUFA biosynthesis compared to a RO-based diet containing only ALA as precursor for n-3 LC-PUFA biosynthesis. In aquaculture nutrition, FA data is frequently presented as % values, but since the crude lipid content was lower in white muscle of seawater fish compared to freshwater fish, % values may not reflect the full nature of FA metabolism. In line with lower lipid content in white muscle of seawater fish, the proportion of TAG to PL was less in seawater fish. PL is rich in EPA and DHA (Sargent et al., 2002; Miller et al., 2006), therefore this might have caused the observed levelling in % n-3 LC-PUFA between freshwater and seawater fish. To verify our observations, absolute values of n-3 LC-PUFA were determined

and provided similar results to % values except for DHA, which was not different for EO and RO fish. However, there was a decreasing trend in the absolute values of EPA, DHA, and n-3 LC-PUFA within dietary treatments between freshwater and seawater fish. This observation again supports the view that a longer growth period is needed to detect any significant differences in n-3 LC-PUFA relative levels and content between fish from freshwater and seawater.

Other studies using dietary EO for different fish species yielded mixed results. In Arctic charr, there was an increase in SDA and ETA in both flesh and liver accompanied by an increase in hepatocyte n-3 LC-PUFA synthesis (Tocher et al., 2006). In Atlantic cod, desaturation of ALA and EPA was very low, but was increased by the presence of dietary EO (Bell et al., 2006). In another study, no apparent increase in biosynthesis was observed along the n-3 biosynthetic pathway when gilthead seabream was fed an EO diet (Diaz-Lopez et al., 2009). It is well known that dietary VO enhance n-3 LC-PUFA biosynthesis and the primary trigger for such an increase in fish fed VO compared to FO is reduced dietary n-3 LC-PUFA (Tocher, 2003). Although different fish species may exhibit dissimilar n-3 LC-PUFA biosynthesis and is generally lower in marine fish species, in these studies fishmeal was not defatted and n-3 LC-PUFA was present in the diet in different proportions as compared to this present experiment where defatting resulted in negligible dietary n-3 LC-PUFA input.

The FA profile, especially n-3 LC-PUFA content of white muscle is very important both for the aquaculture industry and human consumers. Comparable levels of n-3 LC-PUFA could not be achieved between EO and FO fish. However, SDA rich oils can be a better alternative to conventional VO due to higher n-3 LC-PUFA, improved n-3: n-6 ratio and higher total n-3 PUFA in the white muscle. EO was used as model oil in this present study

and is currently considered uneconomic as a substitute for FO (Miller et al., 2008a). The extent to which this research will be useful in providing alternative strategies for replacing fish oil in aquafeeds depends on future advancements in the field of plants genomics to increase synthesis of SDA, increase the n-3: n-6 ratio and ultimately n-3 LC-PUFA in commercially viable oil seed plants (Ursin, 2003).

3.6 CONCLUSION

In the liver, $\Delta 5$ desaturase gene expression was under dietary and environmental regulation, while FA elongase gene expression was regulated by dietary and environment interaction. In both liver and white muscle, $\Delta 6$ desaturase gene expression was under dietary regulation. Over a period of 28 weeks, dietary SDA enriched oil promoted higher n-3 LC-PUFA biosynthesis in the liver and white muscle of Atlantic salmon in both freshwater (112 days) and seawater (84 days) compared to a RO diet. Bypassing of $\Delta 6$ desaturase, availability of extra substrate (SDA) and long nutritional history of the fish on EO diet were identified as reasons for increased n-3 LC-PUFA biosynthesis in the liver and white muscle. However, the increased n-3 LC-PUFA biosynthesis in EO fish was not enough to reach comparable n-3 LC-PUFA levels found in the white muscle of FO fish.

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CHAPTER 4

Restoration of EPA and DHA in rainbow trout, *Oncorhynchus mykiss*, fed a fish oil finishing diet preceded by growth on palm fatty acid distillate diet at optimal and elevated water temperature.

Adapted from Codabaccus, M.B., Ng, W.K., Nichols, P.D., Carter, C.G., 2011. Restoration of EPA and DHA in rainbow trout, *Oncorhynchus mykiss*, fed a fish oil finishing diet preceded by growth on palm fatty acid distillate diet at optimal and elevated water temperature. *In preparation*.

4.1 ABSRACT

Fish oil (FO) replacement in aquafeeds by vegetable oils (VO) for salmonids has become unavoidable due to the rise in the price of FO dictated by the market imbalance between demand and supply. The lack of n-3 long chain polyunsaturated fatty acid (n-3 LC-PUFA) in VO remains a major shortcoming. Feeding fish with a FO finishing diet (FOFD) after grow-out on a VO diet is one strategy to restore the amount of n-3 LC-PUFA in fish, specifically eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The present study investigated for the first time the restoration of EPA and DHA in rainbow trout fed a FOFD for 28 days which was preceded by a grow-out period of 77 days on 50% or 75% palm fatty acid distillate (PFAD) diets (at the expense of added fish oil) at optimal (15°C) or elevated (20°C) water temperatures. The changes in fatty acid (FA) percentage composition of fish fillet and whole carcass were evaluated against a dilution model. Using the FOFD restored EPA and DHA (% total FA) in fillet and whole carcass of fish previously fed the 50% PFAD diet from 85 to 98% of values obtained for fish fed FO diet throughout. In fish previously fed 75% PFAD, using the FOFD restored EPA and DHA to a lesser extent in fillet and whole carcass of fish (from 66 to 93%) compared to fish fed FO diet throughout. Water temperature did not influence the dynamics of EPA and DHA restoration. FA modification after dietary change reflected a dilution of existing FA stores in rainbow trout fillet and whole carcass. The use of the FOFD provided a strategy to restore EPA and DHA following a longer growth period on PFAD-based diet. Growing rainbow trout with diets where 50% and 75% FO was replaced by PFAD followed by a FOFD reduced the use of FO by an estimated average of 30 and 44% respectively at 15°C, and 32 and 48% respectively at 20°C.

4.2 INTRODUCTION

Fish oil (FO) is the main lipid source in feeds of carnivorous farmed fish and aquafeeds use 87% of the global supply of FO, of which over 66% is used for salmonids (Tacon et al., 2006). Driven by a high demand from the rapidly expanding aquaculture industry and static supply from wild caught fisheries, the price of FO has increased considerably in recent years (Naylor et al., 2009). To reduce dependence on FO and to alleviate pressure on wild caught fisheries, vegetable oils (VO) have been evaluated as alternatives for major carnivorous cultured fish species (Bransden et al., 2003; Izquierdo et al., 2005; Montero et al., 2005). The use of palm-based oils, particularly crude palm oil, in aquafeeds for salmonids has previously been investigated as an alternative oil source (Ng et al., 2007). Palm fatty acid distillate (PFAD), a by-product of crude palm oil refining is a cheap source of FA (Bahurmiz and Ng, 2007) and its use in aquafeed for rainbow trout has not been investigated prior to this study, although limited research has been effected on its use in diets for African catfish (*Clarias gariepinus*) (Ng et al., 2004b) and red tilapia hybrid (*Oreochromis* sp.) (Bahurmiz and Ng, 2007). Growth is seldom impaired by substituting FO with VO in salmonid feeds (Rosenlund et al., 2001; Fonseca-Madrigal et al., 2005; Torstensen et al., 2005), however, levels of n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA), in particular eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are reduced, thus decreasing the health promoting benefits to consumers (Seierstad et al., 2005). This dilemma has led to the development of use of a fish oil finishing diet (FOFD) period to restore n-3 LC-PUFA levels in fish after a growth period on VO-based diet. FA changes that occur after changing the diet represent the dilution of existing FA stores, so that the tissue FA composition of fish will resemble that of the diet after a certain feeding period. To this end, a dilution model has been proposed (Robin et al., 2003). Good prediction of tissue FA composition after dietary change based on the dilution model has

been demonstrated (Jobling 2004a; Benedito-Palos et al., 2009). However, deviation from the dilution model for selected FA has been observed (Jobling 2004b, Lane et al., 2006, Turchini et al., 2006). Deviations from the dilution model suggest a combination of dilution, preferential FA metabolism and FA turnover (Jobling 2004b). Preferential FA metabolism is significant for FA restoration because it may accelerate EPA and DHA restoration by preferentially metabolising FA from existing stores (from the previous grow-out diet) rather than from dietary FA. Palm fatty acid distillate (PFAD) is rich in saturated FA (SFA) and monounsaturated FA (MUFA) and is relatively low in C18 PUFA (Bahurmiz and Ng, 2007). This FA composition may favour preferential FA metabolism because SFA and MUFA are preferred substrates for β -oxidation and C18 PUFA, in particular linoleic acid (LA) (typical of most VO) is known to be deposited at a faster rate in fillets and is not easily mobilised after it has been deposited (Turchini et al., 2009). The use of PFAD in aquafeed and subsequent restoration of EPA and DHA by a FOFD for rainbow trout is therefore of interest.

As a result of climate change, elevated temperatures of over 19°C are increasingly encountered in regions where salmonids are cultured in Tasmania, Australia (Miller et al., 2008). Temperature change may influence storage and membrane lipids in salmonids (Hazel et al., 1991). In addition, elevated temperature may positively influence preferential FA metabolism through β -oxidation of existing FA stores. In a previous study, we showed that the apparent digestibility of SFA was improved in rainbow trout fed PFAD-based diets at both optimum and elevated water temperatures (Ng et al., 2010). Therefore, it is important to anticipate for the effects that temperature rise may have on EPA and DHA restoration when using the FOFD strategy. We tested whether a four week FOFD period would restore EPA and DHA in rainbow trout fillet and whole carcass after a grow-out period on diets with different inclusion levels of PFAD at optimal (15°C) or elevated (20°C) water temperatures.

We also investigated whether the dilution model was a good predictor of the modifications in the FA profile of the fillet and whole carcass after the dietary change from PFAD to FO.

4.3 MATERIALS and METHODS

4.3.1 Experimental diet

A FOFD (FO) with the same raw ingredients composition to a FO diet (0% PFAD) (Table 4.1) that was fed during a prior grow-out period (Ng et al., 2010; Appendix) to fish from all three previous dietary treatments containing either: (i) a 50/50 mix of PFAD/FO (termed 50% PFAD), (ii) a 75/25 mix of PFAD/FO (75% PFAD) and (iii) 100% FO (0% PFAD). The FOFD was manufactured into 4 mm pellets using a California Pellet Mill (CL-2), dried and stored at -5°C (Carter et al., 2003). The FA compositions of the FO diet (0%PFAD) used during the grow-out period and the FOFD (FO) are presented in Table 4.2.

Table 4.1: Ingredient and chemical composition (g/kg DM) of rainbow trout FOFD.

	Diet FO
<i>Ingredient composition (g kg⁻¹)</i>	
Fish meal ¹	145
Casein ²	145
Wheat gluten ³	145
Soybean meal ⁴	145
Fish oil ¹	200
Pre-gel starch ⁵	127
Vitamin mix ⁶	7
Mineral mix ⁷	7
Stay-C ⁸	6
Choline chloride ⁹	2
Sipernat ¹⁰	40
CMC ⁹	10
Monobasic calcium phosphate ⁹	20
Yttrium oxide ⁹	1
<i>Chemical composition</i>	
Dry matter (g kg ⁻¹)	912.5
Crude protein	419.8
Total lipid	223.6
Ash	103.2
Energy (MJ kg ⁻¹)	22.4

¹Skretting Australia, Cambridge, Tasmania, Australia; ²MP Biomedicals Australasia Pty. Ltd., Seven Hills NSW, Australia; ³Starch Australasia, Lane Cove, NSW, Australia; ⁴Hamlet Protein A/S, Horstens, Denmark; ⁵Penford Limited, Lane Cove, NSW, Australia; ⁶Vitamin mix (ASV4) as listed in Carter et al. (2003); ⁷Mineral mix (TMV4) as listed in Carter et al. (2003); ⁸L-Ascorbyl-2-polyphosphate (Roche Vitamins Australia, Frenchs Forest, NSW, Australia); ⁹Sigma-Aldrich, Castle Hill, NSW, Australia; ¹⁰Degussa, Frankfurt, Germany.

Table 4.2: Fatty acid composition (% total fatty acids) of the rainbow trout FO diets during grow-out and finishing periods

FA	Diet	
	0% PFAD (grow-out)	FO (finishing)
14:0	6.3	6.5
15:0	0.6	0.5
16:0	21.3	20.5
17:0	0.6	0.6
18:0	4.6	4.5
20:0	0.2	0.2
Other SFA	0.8	1.5
16:1n-7c	8.6	8.6
18:1n-9c	11.2	13.8
18:1n-7c	3.4	4.1
20:1n-9	2.6	3.1
24:1n-9c	0.6	1.9
22:1n-11c	2.1	0.5
Other MUFA	1.8	0.8
18:2n-6	4.6	4.6
20:4n-6	1.0	1.1
16:4n-3	1.2	1.4
18:3n-3	1.1	0.6
18:4n-3	1.8	1.6
20:4n-3	1.0	0.3
20:5n-3	11.1	13.0
22:5n-3	1.8	1.7
22:6n-3	8.2	6.1
Other PUFA	2.8	2.6
Total SFA	34.5	34.3
Total MUFA	30.5	32.8
Total PUFA	34.9	33.0
Total n-3	27.5	24.7
Total n-6	6.2	5.7
n-3: n-6	4.5	4.4

4.3.2 FO finishing diet experiment

The feeding trial was conducted at the University of Tasmania (Launceston, Tasmania, Australia) in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0009762). Rainbow trout (*Onchorynchus mykiss*) were previously fed on the 0% PFAD, 50% PFAD or 75% PFAD diets at 15°C or 20°C for a period of 42

days (Ng et al., 2010) and then in this study for an additional 35 days (total 77 days) before switching to a FOFD (FO) for another 28 days. Fish were reared in a partial recirculation system according to the maintenance protocol described in Carter and Hauler (2000). The experimental system consisted of eighteen 300 L tanks with six treatments and three replicates per treatment in an orthogonal 3x2 factorial design. Fish were initially stocked at 30 fish per tank and were fed on a fixed daily ration of 1.5% body weight.

At the start and end of the FO finishing diet feeding period, fish were bulk weighed and nine fish per treatment (three fish per replicate) were euthanized (100 mg/L, benzocaine) to provide whole carcass samples and a further nine fish per treatment were dissected for the fillet. All fish and tissues were weighed and stored at -20°C until fatty acid analyses.

4.3.3 Chemical analysis

Samples were freeze dried and extracted overnight using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single phase extraction using CHCl₃/MeOH/H₂O (1:2:0.8, v/v/v), followed by phase separation and concentration by rotary evaporation to yield a total lipid extract (TLE).

An aliquot of the TLE was trans-methylated in methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 2 h at 100°C. After addition of MilliQ water (1 ml), the mixture was extracted three times with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). Samples were made up to a known volume with an internal injection standard (19:0 FAME) and analysed by gas chromatography (GC) using an Agilent Technologies 7890A GC (Palo Alto, California, USA) equipped with a Supelco Equity™-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 µm film thickness), an FID, a split/splitless injector and an

Agilent Technologies 7683B Series auto sampler. Helium was used as carrier gas. Samples were injected in splitless mode at an oven temperature of 120°C. After injection, oven temperature was raised to 270°C at 10°C/min and finally to 310°C at 5°C/min. Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, California, USA). GC results are typically subject to an error of up to $\pm 5\%$ of individual component area.

Individual components were identified by mass spectral data and by comparing GC retention time data with those obtained for authentic and laboratory standards. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, Texas, USA). The GC was equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m \times 0.32 mm i.d.) of similar polarity to that described above. Helium was used as carrier gas, with operating conditions described in Miller et al. (2006).

4.3.4 Computation and statistical analysis

Feed consumption (FC) was calculated as the total average amount of dry feed (g) consumed per tank over the number of days of the experiment. Weight gain (WG) was calculated as $WG (\%) = [(W_f - W_i) / W_i] * 100$, where W_i and W_f are initial and final weights respectively. The feed efficiency ratio (FER) was calculated as $FER (g/g) = \text{total weight gain (g)} / FC (g)$. Mean values are reported as \pm standard error of the mean (SEM). The relative levels and absolute amounts of dietary FO and PFAD used per fish was calculated from the average amount of feed consumed per fish during the grow-out and the FOFD periods. Normality and homogeneity of variance were confirmed and percentage data were arcsine transformed prior to analysis. Samples from individual fish were pooled on a tank basis ($n = 3$) and comparison between treatment means for fatty acid composition and growth

performance was by two-way analysis of variance (ANOVA) followed by multiple comparison using Tukey-Kramer HSD wherever applicable. Significance was accepted at probabilities $P < 0.05$.

The dilution model is expressed as follows (Robin et al., 2003):

$$P_t = P_r + (P_i - P_r)/(Q_t - Q_i)$$

where P_t was defined as the percentage of fatty acid in the whole carcass and fillet of fish previously fed at different dietary levels of PFAD at time t , P_i is the initial percentage of fatty acid in the whole carcass and fillet of fish previously fed at different levels of PFAD and P_r is the percentage of fatty acid in the whole carcass and fillet of fish fed FO throughout at time t . Q_i is the initial total amount of lipid present and Q_t is the amount present at time t in the whole carcass and fillet of fish previously fed at different levels of PFAD. Total lipid content was determined gravimetrically following extraction of tissues using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). A regression analysis between predicted (from the dilution model) and observed FA values was performed and individual regression lines were compared to the line of equity (Jobling, 2004b). Analysis of covariance (ANCOVA) was used to compare slopes and intercept of regression lines to the line of equity. Statistical analysis was performed using SPSS for Windows version 16.0.

4.4 RESULTS

4.4.1 Growth

After 77 days on the grow-out diets, and prior to feeding FO, fish fed on 75%PFAD had lower body weight than fish fed on 0%PFAD and 50%PFAD at 15°C (Table 4.3). The final weight of fish after feeding FOFD was dependent on their starting body weight after 11 weeks on the various dietary treatments (Table 4.3). At 15°C, fish fed FO diet (0%

PFAD/FO) throughout had the highest final weight though not statistically different from fish previously fed 50%PFAD (50% PFAD/FO). There were no significant ($P > 0.05$) differences in WG (%) between fish previously fed different levels of PFAD and fish fed FO throughout (0% PFAD/FO), at both water temperatures. Fish grown at 15°C had higher WG, FC and FER than fish grown at 20°C.

Table 4.3: Growth and efficiencies of rainbow trout previously fed on diets differing in % PFAD for 77 days and then fed on FO for 28 days at two water temperatures

Temp	Diet	Wi (g)	Wf (g)	WG (%)	FC	FER
15°C	0%PFAD/FO	387.4±13.9 ^z	510.2±22.8 ^z	31.6±1.5	159.2±5.2 ^z	0.8±0.1
	50%PFAD/FO	350.2±6.2 ^{yz}	465.0±8.3 ^{yz}	32.8±1.6	144.0±1.4 ^{yz}	0.8±0.1
	75%PFAD/FO	322.6±5.9 ^y	431.8±3.3 ^y	33.9±2.2	139.2±2.0 ^y	0.8±0.1
20°C	0%PFAD/FO	282.6±6.1 ^x	344.5±4.2 ^x	22.0±1.4*	112.3±2.6 ^x	0.6±0.1*
	50%PFAD/FO	285.9±4.8 ^x	357.5±8.5 ^x	25.0±1.7*	114.1±2.6 ^x	0.6±0.1*
	75%PFAD/FO	272.3±2.4 ^x	340.2±8.4 ^x	24.9±2.1*	109.3±1.9 ^x	0.6±0.1*
Effects	Diet	$F=12.9, P<0.05$	$F=6.8, P<0.05$	$F=1.2, P=0.33$	$F=11.2, P<0.05$	$F=1.5, P=0.26$
	Temp	$F=157.1, P<0.05$	$F=194.1, P<0.05$	$F=36.8, P<0.05$	$F=175.1, P<0.05$	$F=33.7, P<0.05$
	Interaction	$F=6.8, P<0.05$	$F=5.7, P<0.05$	$F=0.1, P=0.87$	$F=7.6, P<0.05$	$F=0.5, P=0.63$

W_i, initial weight (at start of FO finishing diet); W_f, final weight; WG, weight gain; FC, total amount of dry feed consumed per tank over the experiment period; FER, feed efficiency ratio. 0% PFAD/FO – fish fed on FO diet throughout, 50% PFAD/FO – fish fed on 50% PFAD then on FO, 75%PFAD/FO – fish fed on 75% PFAD then on FO. Values are means ± SEM, $n = 3$. Means in a column followed by different superscript letters ^{x, y, z} denotes dietary and temperature interaction. An * represents a temperature difference (Tukey's test $P < 0.05$).

4.4.2 EPA, DHA and n-3: n-6 ratio – Initial samples (after grow-out)

There was a higher percentage of EPA, DHA and the n-3: n-6 ratio in the fillet of 0% PFAD fish compared to the fillets of 50% and 75% PFAD fish (Table 4.4). Higher percentages of EPA and DHA were obtained for fish at 20°C compared to fish at 15°C.

There was a higher percentage of EPA and DHA and a higher n-3: n-6 ratio in the whole carcass of 0% PFAD fish compared to the whole carcass of 50% and 75% PFAD fish (Table 4.4). A higher percentage of EPA was obtained for fish at 20°C compared to fish at 15°C.

4.4.3 Fatty acid composition (%) – fillet

There were no significant ($P > 0.05$) differences in percentage total SFA, total MUFA and total PUFA in the fillet between fish previously fed 50% PFAD (50% PFAD/FO) and 0% PFAD/FO fish (Table 4.5). Total SFA and total PUFA was significantly ($P < 0.05$) lower in 75% PFAD/FO fish compared to the other two treatments. EPA and DHA in the fillet did not differ between 0% PFAD/FO and 50% PFAD/FO fish, while lower levels were obtained for 75% PFAD/FO fish. The results indicated a high degree of restoration of EPA and DHA in the fillet for 50% PFAD/FO fish; 97% and 89% for EPA and 91% and 98% for DHA at 15°C and 20°C, respectively. For 75% PFAD/FO fish, restoration was reduced - EPA restoration was 85% and 68% and DHA restoration was 78% and 66% at 15°C and 20°C, respectively. There was a significant ($P < 0.05$) temperature difference in lipid content of fillet with more lipid at 15°C than at 20°C.

4.4.4 Fatty acid composition (%) – whole carcass

Whole carcass FA profiles of fish followed the same pattern observed for fillet FA profiles and differences were predominantly of dietary origin (Table 4.6). There were no significant ($P > 0.05$) differences in total SFA and total MUFA between 50% PFAD/FO fish and 0% PFAD/FO fish. Total SFA was significantly ($P < 0.05$) less in 75% PFAD/FO fish compared to the other two treatments, while total PUFA did not differ between diets. There was no significant difference in EPA and DHA between 50% PFAD/FO fish and 0%

PFAD/FO fish. DHA was not significantly different in 75% PFAD/FO fish compared to the other two treatments, while EPA was lower. The results again indicate a large restoration in EPA and DHA for fish fed 50% PFAD/FO; 89% and 93% for EPA and 85% and 86% for DHA at 15°C and 20°C, respectively. For 75% PFAD/FO fish, restoration was again reduced particularly at 20°C - EPA restoration was 91% and 72% and DHA restoration was 93% and 76% at 15°C and 20°C, respectively. There was no significant difference for lipid content in the whole carcass between fish fed different levels of PFAD followed by FO at both temperatures.

Table 4.4: Percent composition of EPA and DHA and n-3: n-6 ratio of initial fillet and whole carcass samples of rainbow trout (after grow-out with diets differing in PFAD level) at two water temperatures

FA	15°C			20°C		
	0%PFAD	50%PFAD	75%PFAD	0%PFAD	50%PFAD	75%PFAD
Fillet						
20:5n-3	6.5 ± 0.2 ^c	4.9 ± 0.1 ^b	3.0 ± 0.1 ^a	7.1 ± 0.1 ^{c*}	5.4 ± 0.0 ^{b*}	3.5 ± 0.1 ^{a*}
22:6n-3	13.2 ± 0.8 ^c	10.6 ± 0.4 ^b	8.0 ± 0.3 ^a	13.5 ± 0.4 ^{c*}	11.4 ± 0.4 ^{b*}	9.6 ± 0.2 ^{a*}
n-3: n-6	4.1 ± 0.0 ^c	1.9 ± 0.0 ^b	1.1 ± 0.0 ^a	4.0 ± 0.2 ^c	2.0 ± 0.0 ^b	1.4 ± 0.0 ^a
Whole Carcass						
20:5n-3	5.6 ± 0.1 ^c	4.4 ± 0.1 ^b	3.0 ± 0.3 ^a	6.5 ± 0.1 ^{c*}	4.8 ± 0.1 ^{b*}	3.0 ± 0.2 ^{a*}
22:6n-3	9.0 ± 0.2 ^c	7.7 ± 0.1 ^b	5.8 ± 0.3 ^a	9.8 ± 0.1 ^c	7.6 ± 0.1 ^b	5.9 ± 0.3 ^a
n-3: n-6	3.0 ± 0.1 ^c	1.6 ± 0.0 ^b	1.0 ± 0.1 ^a	3.0 ± 0.0 ^c	1.6 ± 0.0 ^b	1.1 ± 0.1 ^a

Values are means ± SEM, $n = 3$. Means in a row followed by different superscript letters ^{a, b, c}, denotes dietary differences. An * represents a temperature difference (Tukey's test, $P < 0.05$). 0% PFAD/FO – fish fed on FO diet throughout, 50% PFAD/FO – fish fed on 50% PFAD then on FO, 75%PFAD/FO – fish fed on 75% PFAD then on FO.

Table 4.5: Fatty acid composition (% of total FA) and lipid content (mg/g) of fillet of rainbow trout fed a FO finishing diet for 28 days after 77 days growth period on diets differing in PFAD level at two water temperatures.

FA	15°C			20°C		
	0%PFAD/FO	50%PFAD/FO	75%PFAD/FO	0%PFAD/FO	50%PFAD/FO	75%PFAD/FO
14:0	4.5 ± 0.1 ^z	4.3 ± 0.1 ^{xy}	3.4 ± 0.1 ^x	4.9 ± 0.2 ^z	3.7 ± 0.1 ^{xy}	3.7 ± 0.2 ^{xy}
16:0	21.1 ± 0.3	21.6 ± 0.2	20.7 ± 0.6	22.2 ± 0.1*	22.5 ± 0.4*	21.6 ± 0.4*
17:0	0.4 ± 0.0 ^b	0.4 ± 0.0 ^{ab}	0.3 ± 0.0 ^a	0.4 ± 0.0 ^b	0.3 ± 0.0 ^{ab}	0.2 ± 0.1 ^a
18:0	5.2 ± 0.0 ^b	4.9 ± 0.3 ^{ab}	4.6 ± 0.1 ^a	5.2 ± 0.1 ^b	4.8 ± 0.2 ^{ab}	4.4 ± 0.1 ^a
Other SFA ¹	1.5 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.5 ± 0.2	1.3 ± 0.0	1.2 ± 0.1
16:1n-7c	8.4 ± 0.2 ^b	7.4 ± 0.4 ^a	6.8 ± 0.3 ^a	8.0 ± 0.4 ^b	7.0 ± 0.3 ^a	7.0 ± 0.2 ^a
18:1n-9c	19.8 ± 0.2 ^a	23.3 ± 1.0 ^b	28.3 ± 1.2 ^c	18.5 ± 0.4 ^a	22.8 ± 0.3 ^b	28.4 ± 1.3 ^c
18:1n-7c	4.6 ± 0.1 ^b	4.0 ± 0.0 ^a	3.9 ± 0.0 ^a	4.4 ± 0.0 ^b	4.0 ± 0.1 ^a	4.0 ± 0.1 ^a
20:1n-9	3.7 ± 0.1 ^c	2.4 ± 0.1 ^a	2.9 ± 0.0 ^b	3.2 ± 0.2 ^{c*}	2.4 ± 0.1 ^{a*}	2.7 ± 0.1 ^{b*}
20:1n-7c	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
24:1n-9c	0.5 ± 0.0 ^b	0.4 ± 0.0 ^a	0.3 ± 0.0 ^a	0.5 ± 0.1 ^b	0.5 ± 0.0 ^a	0.4 ± 0.0 ^a
Other MUFA ²	2.3 ± 0.1 ^b	1.4 ± 0.0 ^a	1.6 ± 0.1 ^a	2.0 ± 0.1 ^{b*}	1.3 ± 0.1 ^{a*}	1.3 ± 0.1 ^{a*}
18:2n-6	4.3 ± 0.2 ^a	6.4 ± 0.4 ^b	6.6 ± 0.4 ^b	4.4 ± 0.4 ^a	6.1 ± 0.1 ^b	7.1 ± 0.2 ^b
20:4n-6	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.0
Other n-6 ³	2.6 ± 0.1	2.3 ± 0.2	2.2 ± 0.1	2.0 ± 0.1	2.2 ± 0.1	2.2 ± 0.3
20:5n-3	6.7 ± 0.2 ^b	6.5 ± 0.3 ^b	5.5 ± 0.4 ^a	7.4 ± 0.4 ^b	6.6 ± 0.2 ^b	5.0 ± 0.2 ^a
22:6n-3	9.7 ± 0.5 ^b	8.8 ± 0.8 ^b	7.6 ± 0.3 ^a	10.4 ± 1.1 ^b	10.2 ± 0.4 ^b	6.9 ± 0.8 ^a
Other n-3 ⁴	3.4 ± 0.2 ^b	3.2 ± 0.2 ^b	2.6 ± 0.2 ^a	3.5 ± 0.2 ^b	3.0 ± 0.0 ^b	2.6 ± 0.1 ^a
Total SFA	32.6 ± 0.3 ^b	32.4 ± 0.2 ^b	30.2 ± 0.6 ^a	34.1 ± 0.3 ^{b*}	32.6 ± 0.6 ^{b*}	31.2 ± 0.4 ^{a*}
Total MUFA	39.8 ± 0.1 ^a	39.4 ± 1.1 ^a	44.2 ± 1.2 ^b	37.1 ± 1.0 ^a	38.3 ± 0.5 ^a	44.2 ± 1.7 ^b
Total PUFA	28.0 ± 0.4 ^b	28.5 ± 1.0 ^b	25.9 ± 0.8 ^a	29.1 ± 1.1 ^b	29.4 ± 0.4 ^b	24.9 ± 1.3 ^a
Total n-3	19.7 ± 0.5 ^b	18.5 ± 1.3 ^b	15.8 ± 0.9 ^a	21.4 ± 1.3 ^b	19.8 ± 0.4 ^b	14.5 ± 1.2 ^a
Total n-6	7.7 ± 0.1 ^a	9.6 ± 0.2 ^b	9.7 ± 0.4 ^b	7.3 ± 0.3 ^a	9.2 ± 0.1 ^b	10.0 ± 0.2 ^b
n-3: n-6	2.6 ± 0.1 ^a	1.9 ± 0.2 ^b	1.6 ± 0.1 ^c	3.0 ± 0.3 ^a	2.2 ± 0.1 ^b	1.4 ± 0.1 ^c
Lipid content ⁵						
Wet	42.3 ± 5.1	36.0 ± 3.3	43.4 ± 3.8	26.5 ± 5.8*	27.9 ± 2.8*	37.9 ± 6.0*
Dry	150.1 ± 17.7	133.2 ± 12.9	153.8 ± 9.9	100.3 ± 22.0*	103.6 ± 10.8*	137.4 ± 18.3*

Values are means ± SEM, $n = 3$. Means in a row followed by different superscript letters ^{a, b, c} denotes differences between diets. Means in a row followed by different superscript letters ^{x, y, z} denotes dietary and temperature interaction. An * represents a temperature difference (Tukey's test, $P < 0.05$). 0% PFAD/FO – fish fed on FO diet throughout, 50% PFAD/FO – fish fed on 50% PFAD then on FO, 75% PFAD/FO – fish fed on 75% PFAD then on FO. ¹Includes 15:0, 21:0 and 24:0. ²Includes 16:1n-9, 16:1n-5, 17:1, 17:1n-8, 18:1n-5, 22:1n-9, 22:1n-7, 22:1n-11 and 24:1n-7. ³Includes 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:2n-6, 22:4n-6 and 22:5n-6. ⁴Includes 16:3, 16:4n-3, 18:4n-3, 20:4n-3, 21:5n-3, 22:4n-3, 24:5n-3 and 24:6n-3. ⁵Determined gravimetrically.

Table 4.6: Fatty acid composition (% of total FA) and lipid content (mg/g) of whole carcass of rainbow trout fed a FO finishing diet for 28 days after 77 days growth period on diets differing in PFAD level at two water temperatures.

FA	15°C			20°C		
	0%PFAD/FO	50%PFAD/FO	75%PFAD/FO	0%PFAD/FO	50%PFAD/FO	75%PFAD/FO
14:0	4.9 ± 0.2 ^b	4.1 ± 0.4 ^{ab}	3.2 ± 0.1 ^a	5.1 ± 0.7 ^b	4.1 ± 0.3 ^{ab}	4.0 ± 0.2 ^a
16:0	20.8 ± 0.6 ^{ab}	21.1 ± 0.7 ^b	19.2 ± 0.2 ^a	21.1 ± 0.5 ^{ab}	21.7 ± 0.5 ^b	20.7 ± 0.5 ^a
17:0	0.4 ± 0.0 ^b	0.3 ± 0.0 ^a	0.3 ± 0.0 ^a	0.6 ± 0.0 ^{b*}	0.4 ± 0.0 ^{a*}	0.4 ± 0.0 ^{a*}
18:0	4.9 ± 0.2 ^b	4.8 ± 0.2 ^b	4.4 ± 0.1 ^a	5.0 ± 0.0 ^b	4.7 ± 0.1 ^b	4.2 ± 0.1 ^a
Other SFA ¹	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.0	1.2 ± 0.2	1.1 ± 0.0
16:1n-7c	9.2 ± 0.4 ^c	8.2 ± 0.2 ^b	7.3 ± 0.3 ^a	8.9 ± 0.1 ^{c*}	7.6 ± 0.1 ^{b*}	6.6 ± 0.3 ^{a*}
18:1n-9c	22.6 ± 0.7 ^a	27.9 ± 0.7 ^b	30.0 ± 1.7 ^c	21.5 ± 0.1 ^a	27.6 ± 1.1 ^b	32.6 ± 0.4 ^c
18:1n-7c	4.8 ± 0.2 ^b	4.4 ± 0.1 ^a	4.1 ± 0.1 ^a	4.9 ± 0.1 ^b	4.2 ± 0.1 ^a	4.0 ± 0.1 ^a
20:1n-9	3.9 ± 0.2 ^c	2.6 ± 0.1 ^a	3.2 ± 0.1 ^b	3.7 ± 0.2 ^{c*}	2.3 ± 0.1 ^{a*}	2.7 ± 0.1 ^{b*}
20:1n-7c	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.0
22:1n-11c	1.3 ± 0.1 ^{yz}	1.0 ± 0.1 ^{xy}	0.9 ± 0.1 ^{xy}	1.7 ± 0.2 ^z	1.0 ± 0.1 ^{xy}	0.7 ± 0.0 ^x
24:1n-9c	0.5 ± 0.0 ^b	0.3 ± 0.0 ^a	0.4 ± 0.0 ^a	0.5 ± 0.0 ^b	0.3 ± 0.1 ^a	0.3 ± 0.0 ^a
Other MUFA ²	1.3 ± 0.2 ^b	0.5 ± 0.2 ^a	0.7 ± 0.1 ^a	1.1 ± 0.2 ^b	0.2 ± 0.2 ^a	0.6 ± 0.0 ^a
18:2n-6	5.4 ± 0.2 ^a	7.0 ± 0.1 ^b	7.1 ± 0.3 ^b	5.7 ± 0.1 ^{a*}	7.8 ± 0.2 ^{b*}	8.1 ± 0.1 ^{b*}
20:4n-6	0.7 ± 0.0	0.6 ± 0.1	0.8 ± 0.0	0.7 ± 0.0	0.5 ± 0.2	0.6 ± 0.0
Other n-6 ³	0.2 ± 0.2 ^a	0.6 ± 0.1 ^{ab}	1.0 ± 0.2 ^b	0.3 ± 0.2 ^a	0.3 ± 0.3 ^{ab}	0.7 ± 0.3 ^b
18:4n-3	1.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
20:5n-3	5.6 ± 0.2 ^b	5.0 ± 0.6 ^{ab}	5.1 ± 0.3 ^a	5.8 ± 0.2 ^b	5.4 ± 0.1 ^{ab}	4.2 ± 0.1 ^a
22:5n-3	1.8 ± 0.1 ^{bc}	1.5 ± 0.1 ^a	1.6 ± 0.1 ^a	1.9 ± 0.1 ^b	1.6 ± 0.1 ^a	1.3 ± 0.1 ^a
22:6n-3	6.7 ± 0.5	5.7 ± 0.6	6.2 ± 0.4	6.6 ± 0.4	5.7 ± 0.4	5.0 ± 0.3
Other n-3 ⁴	1.5 ± 0.1 ^y	1.1 ± 0.1 ^{xy}	1.3 ± 0.3 ^{xy}	1.2 ± 0.1 ^{xy}	1.5 ± 0.3 ^y	0.6 ± 0.2 ^x
Total SFA	32.2 ± 1.1 ^b	31.4 ± 0.8 ^b	28.3 ± 0.3 ^a	33.1 ± 1.2 ^b	32.1 ± 0.3 ^b	30.3 ± 0.2 ^a
Total MUFA	44.0 ± 0.4 ^a	45.4 ± 1.0 ^a	47.0 ± 1.0 ^b	42.8 ± 0.1 ^a	43.5 ± 0.8 ^a	47.8 ± 0.2 ^b
Total PUFA	23.8 ± 1.5	23.2 ± 1.7	24.7 ± 0.7	24.1 ± 1.1	24.4 ± 0.9	21.9 ± 0.3
Total n-3	16.9 ± 1.0 ^b	14.5 ± 1.4 ^{ab}	15.3 ± 1.0 ^a	16.8 ± 0.9 ^b	15.4 ± 0.6 ^{ab}	12.2 ± 0.5 ^a
Total n-6	6.4 ± 0.5 ^a	8.2 ± 0.3 ^b	9.0 ± 0.4 ^b	6.7 ± 0.2 ^a	8.6 ± 0.4 ^b	9.4 ± 0.2 ^b
n-3: n-6	2.7 ± 0.1 ^b	1.8 ± 0.1 ^a	1.7 ± 0.2 ^a	2.5 ± 0.1 ^{b*}	1.8 ± 0.0 ^{a*}	1.3 ± 0.1 ^{a*}
Lipid content ⁵						
Wet	152.8 ± 3.4	149.5 ± 2.3	142.1 ± 5.3	142.5 ± 4.8	132.5 ± 18.7	156.6 ± 5.8
Dry	414.4 ± 12.7	417.1 ± 12.7	387.7 ± 26.9	413.1 ± 7.6	401.7 ± 48.9	430.2 ± 14.3

Values are means ± SEM, $n = 3$. Means in a row followed by different superscript letters ^{a, b, c} denotes differences between diets. Means in a row followed by different letters ^{x, y, z} denotes dietary and temperature interaction. An * represents a temperature difference (Tukey's test, $P < 0.05$). 0% PFAD/FO – fish fed on FO diet throughout, 50% PFAD/FO – fish fed on 50% PFAD then on FO, 75%PFAD/FO – fish fed on 75% PFAD then on FO. ¹Includes 15:0, 21:0 and 24:0. ²Includes 16:1n-9, 16:1n-5, 17:1, 17:1n-8, 18:1n-5, 22:1n-9, 22:1n-11 and 24:1n-7. ³Includes 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. ⁴Includes 16:4n-3, 18:4n-3, 20:4n-3, 21:5n-3 and 24:6n-3. ⁵Determined gravimetrically.

Table 4.7: Summary of Two-Way ANOVA on FA (%) composition and lipid content (mg/g) of liver, fillet and whole carcass in rainbow trout fed a FO finishing diet for 28 days after a 77 days growth period on diets differing in PFAD level.

FA	Diet	Effect Liver			Diet	Effect fillet		Effect whole carcass		
		Temp	Interaction			Temp	Interaction	Diet	Temp	Interaction
14:0	$F=0.3, P=0.74$	$F=6.0, P<0.05$	$F=2.3, P=0.14$	$F=28.4, P<0.05$	$F=0.0, P=0.85$	$F=7.1, P<0.05$	$F=6.8, P<0.05$	$F=1.4, P=0.26$	$F=0.5, P=0.65$	
16:0	$F=0.7, P=0.50$	$F=0.1, P=0.79$	$F=0.2, P=0.80$	$F=3.0, P=0.09$	$F=10.0, P<0.05$	$F=0.1, P=0.92$	$F=4.2, P<0.05$	$F=3.6, P=0.08$	$F=0.6, P=0.57$	
17:0	$F=0.3, P=0.77$	$F=2.63, P=0.13$	$F=0.4, P=0.70$	$F=5.5, P<0.05$	$F=0.3, P=0.59$	$F=0.4, P=0.71$	$F=12.0, P<0.05$	$F=13.7, P<0.05$	$F=1.7, P=0.22$	
18:0	$F=0.4, P=0.68$	$F=44.3, P<0.05$	$F=0.5, P=0.65$	$F=8.3, P<0.05$	$F=0.5, P=0.49$	$F=0.2, P=0.79$	$F=12.9, P<0.05$	$F=0.2, P=0.66$	$F=1.1, P=0.37$	
Other SFA	NA	NA	NA	$F=3.9, P=0.05$	$F=0.5, P=0.51$	$F=0.29, P=0.75$	$F=2.6, P=0.11$	$F=0.0, P=0.94$	$F=0.1, P=0.88$	
16:1n-7c	$F=0.7, P=0.53$	$F=18.9, P<0.05$	$F=0.3, P=0.72$	$F=10.5, P<0.05$	$F=0.8, P=0.39$	$F=0.6, P=0.57$	$F=30.8, P<0.05$	$F=6.3, P<0.05$	$F=0.4, P=0.68$	
18:1n-9c	$F=0.1, P=0.89$	$F=28.0, P<0.05$	$F=0.1, P=0.89$	$F=58.9, P<0.05$	$F=0.7, P=0.44$	$F=0.3, P=0.75$	$F=49.1, P<0.05$	$F=0.3, P=0.60$	$F=2.1, P=0.16$	
18:1n-7c	$F=1.3, P=0.31$	$F=8.0, P<0.05$	$F=0.4, P=0.68$	$F=44.2, P<0.05$	$F=0.3, P=0.57$	$F=1.9, P=0.20$	$F=19.1, P<0.05$	$F=0.7, P=0.43$	$F=0.6, P=0.57$	
20:1n-9	$F=1.4, P=0.29$	$F=0.0, P=0.90$	$F=2.5, P=0.13$	$F=35.8, P<0.05$	$F=4.9, P<0.05$	$F=2.0, P=0.18$	$F=43.1, P<0.05$	$F=7.6, P<0.05$	$F=0.4, P=0.68$	
20:1n-7c	$F=0.1, P=0.88$	$F=0.2, P=0.68$	$F=1.1, P=0.35$	$F=0.6, P=0.55$	$F=2.2, P=0.16$	$F=1.3, P=0.31$	$F=3.4, P=0.05$	$F=2.8, P=0.12$	$F=0.3, P=0.74$	
22:1n-11c	NA	NA	NA	NA	NA	NA	$F=27.9, P<0.05$	$F=0.7, P=0.41$	$F=4.1, P<0.05$	
24:1n-9c	$F=1.5, P=0.26$	$F=43.0, P<0.05$	$F=4.7, P<0.05$	$F=6.6, P<0.05$	$F=0.9, P=0.36$	$F=0.7, P=0.52$	$F=6.6, P<0.05$	$F=0.9, P=0.36$	$F=0.7, P=0.52$	
Other MUFA	NA	NA	NA	$F=10.6, P<0.05$	$F=1.3, P=0.28$	$F=0.3, P=0.74$	$F=10.6, P<0.05$	$F=1.3, P=0.28$	$F=0.3, P=0.74$	
18:2n-6	$F=2.3, P=0.14$	$F=1.9, P=0.20$	$F=2.0, P=0.17$	$F=68.1, P<0.05$	$F=20.0, P<0.05$	$F=1.4, P=0.30$	$F=68.1, P<0.05$	$F=20.0, P<0.05$	$F=1.4, P=0.30$	
20:4n-6	$F=0.5, P=0.59$	$F=12.0, P<0.05$	$F=0.3, P=0.78$	$F=1.7, P=0.23$	$F=1.4, P=0.26$	$F=0.2, P=0.82$	$F=1.7, P=0.23$	$F=1.4, P=0.26$	$F=0.2, P=0.82$	
Other n-6	NA	NA	NA	$F=4.3, P<0.05$	$F=1.3, P=0.27$	$F=0.6, P=0.56$	$F=4.3, P<0.05$	$F=1.3, P=0.27$	$F=0.6, P=0.56$	
18:4n-3	NA	NA	NA	NA	NA	NA	$F=2.6, P=0.11$	$F=0.1, P=0.80$	$F=0.1, P=0.90$	
20:5n-3	$F=0.7, P=0.5$	$F=19.5, P<0.05$	$F=0.2, P=0.84$	$F=19.2, P<0.05$	$F=0.2, P=0.69$	$F=2.4, P=0.13$	$F=6.2, P<0.05$	$F=0.1, P=0.80$	$F=2.8, P=0.10$	
22:5n-3	NA	NA	NA	NA	NA	NA	$F=6.6, P=0.16$	$F=0.1, P=0.80$	$F=2.1, P=0.16$	
22:6n-3	$F=0.8, P=0.47$	$F=60.3, P<0.05$	$F=0.9, P=0.42$	$F=8.5, P<0.05$	$F=0.7, P=0.43$	$F=1.1, P=0.35$	$F=3.3, P=0.07$	$F=1.8, P=0.21$	$F=1.1, P=0.38$	
Other n-3	NA	NA	NA	$F=12.3, P<0.05$	$F=0.2, P=0.70$	$F=0.6, P=0.60$	$F=2.8, P=0.10$	$F=1.9, P=0.20$	$F=1.3, P=0.31$	
Total SFA	$F=0.4, P=0.71$	$F=8.6, P<0.05$	$F=0.1, P=0.89$	$F=19.2, P<0.05$	$F=6.3, P<0.05$	$F=1.0, P=0.41$	$F=10.5, P<0.05$	$F=3.7, P=0.08$	$F=5.2, P<0.05$	
Total MUFA	$F=0.1, P=0.94$	$F=19.3, P<0.05$	$F=0.0, P=0.97$	$F=18.1, P<0.05$	$F=2.1, P=0.17$	$F=0.8, P=0.49$	$F=17.7, P<0.05$	$F=1.9, P=0.20$	$F=4.8, P<0.05$	
Total PUFA	$F=0.6, P=0.55$	$F=30.3, P<0.05$	$F=0.3, P=0.74$	$F=9.2, P<0.05$	$F=0.2, P=0.66$	$F=0.8, P=0.49$	$F=0.2, P=0.86$	$F=0.2, P=0.66$	$F=0.6, P=0.58$	
Total n-3	$F=0.7, P=0.52$	$F=43.6, P<0.05$	$F=0.5, P=0.63$	$F=15.8, P<0.05$	$F=0.4, P=0.54$	$F=1.4, P=0.30$	$F=5.6, P<0.05$	$F=1.0, P=0.35$	$F=0.1, P=0.93$	
Total n-6	$F=0.3, P=0.73$	$F=4.7, P=0.05$	$F=0.1, P=0.91$	$F=54.9, P<0.05$	$F=0.8, P=0.38$	$F=2.0, P=0.18$	$F=30.8, P<0.05$	$F=2.1, P=0.18$	$F=2.1, P=0.16$	
n-3: n-6	$F=0.5, P=0.64$	$F=14.9, P<0.05$	$F=0.5, P=0.60$	$F=30.1, P<0.05$	$F=1.2, P=0.30$	$F=2.0, P=0.18$	$F=57.5, P<0.05$	$F=4.9, P<0.05$	$F=1.6, P=0.25$	
Lipid content										
Wet	$F=0.2, P=0.81$	$F=3.5, P=0.09$	$F=1.0, P=0.40$	$F=1.9, P=0.20$	$F=6.6, P<0.05$	$F=0.7, P=0.54$	$F=0.5, P=0.61$	$F=0.4, P=0.56$	$F=1.8, P=0.20$	
Dry	$F=0.4, P=0.71$	$F=3.1, P=0.11$	$F=1.3, P=0.30$	$F=1.6, P=0.24$	$F=6.1, P<0.05$	$F=0.6, P=0.60$	$F=0.0, P=0.98$	$F=0.2, P=0.68$	$F=0.8, P=0.49$	

4.4.5 Dietary FO and PFAD consumed per fish

During the duration of the whole experiment (grow-out and FOFD), 50% PFAD/FO fish at 15°C consumed on average 48 g and 21 g of FO and PFAD respectively representing 70% FO and 30% PFAD dietary use (Fig. 4.1a & b). For 50% PFAD/FO fish at 20°C, FO and PFAD consumption were 41 g and 19 g respectively, which represent 68% FO and 32% PFAD dietary use. For 75% PFAD/FO fish at 15°C, FO and PFAD consumption were 35 g and 29 g respectively, which represent 56% FO and 44% PFAD dietary use. For 75% PFAD/FO fish at 20°C, FO and PFAD consumption were 30g and 27g respectively representing 52% FO and 48% PFAD dietary use.

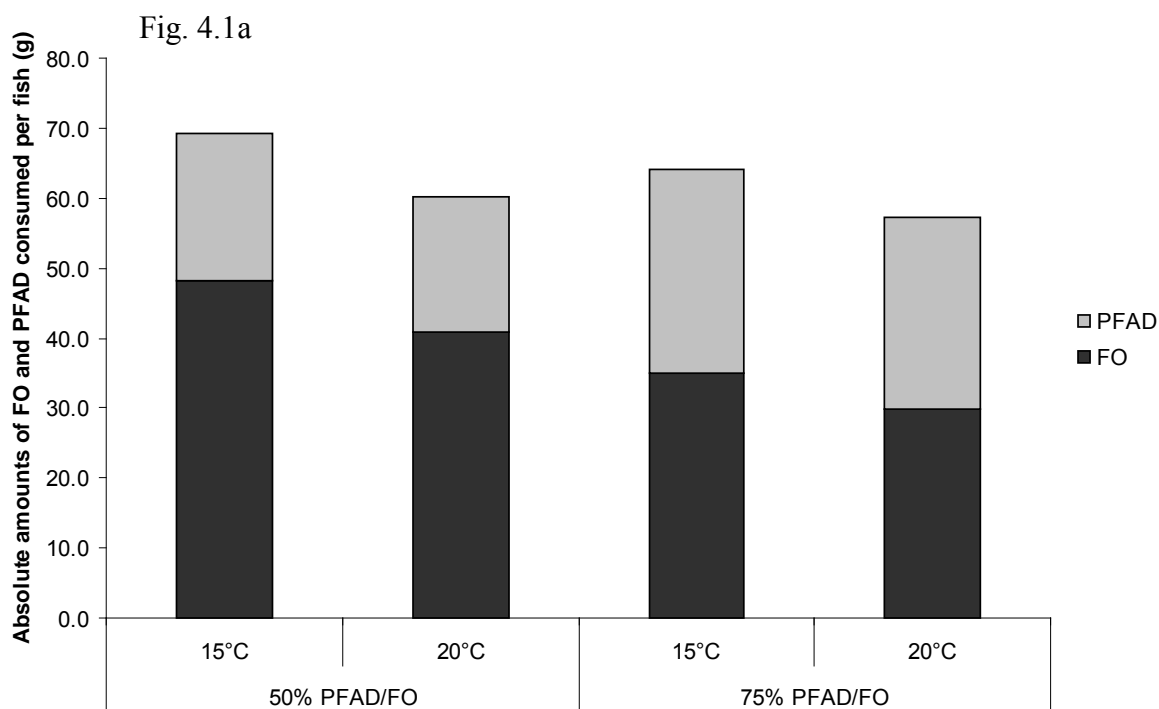


Fig. 4.1b

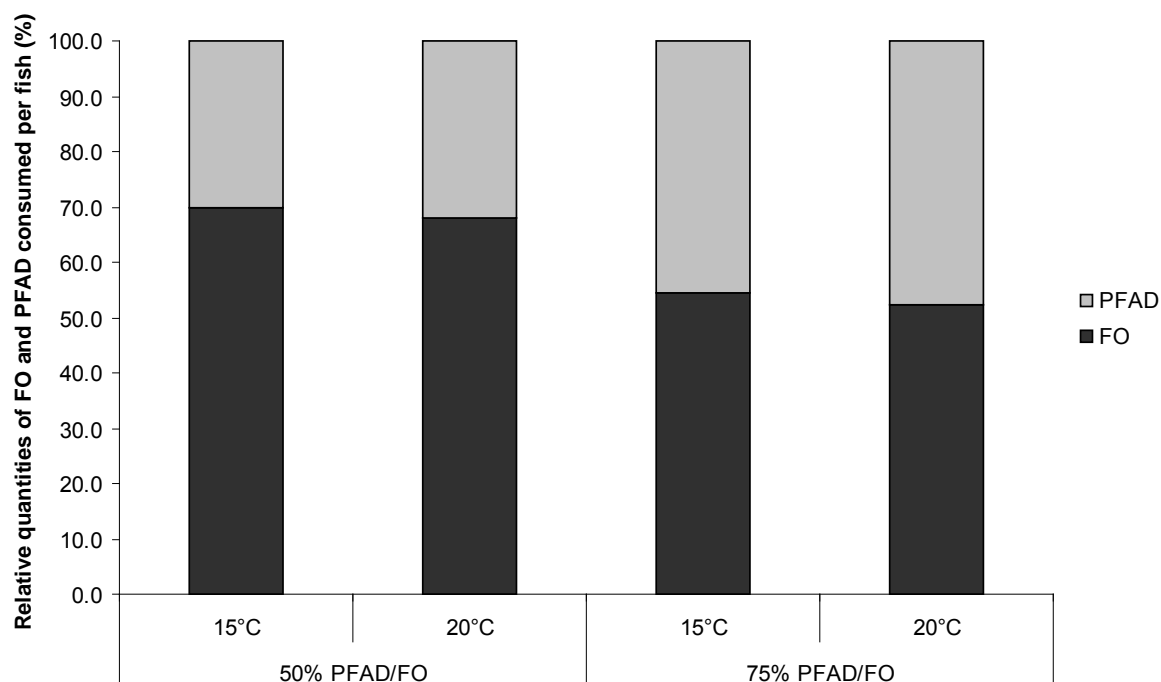


Figure 4.1: Dietary FO (fish oil) and PFAD (palm fatty acid distillate) consumed per fish during the combined grow-out and FOFD period, (a) absolute amounts (g) (b) relative levels (% of total FA).

4.4.6 Absolute amounts of EPA and DHA (mg/g) – fillet

Comparison after the dietary shift to FO of fillet EPA and DHA content in absolute amounts (mg/g basis) gave contrasting results to that observed for %FA values. There were no dietary differences in EPA and DHA content between fish previously fed on different levels of PFAD compared to 0%PFAD/FO. A temperature difference ($P < 0.05$) was observed, with higher EPA and DHA content in fillet of the fish grown at 15°C (Fig 4.2).

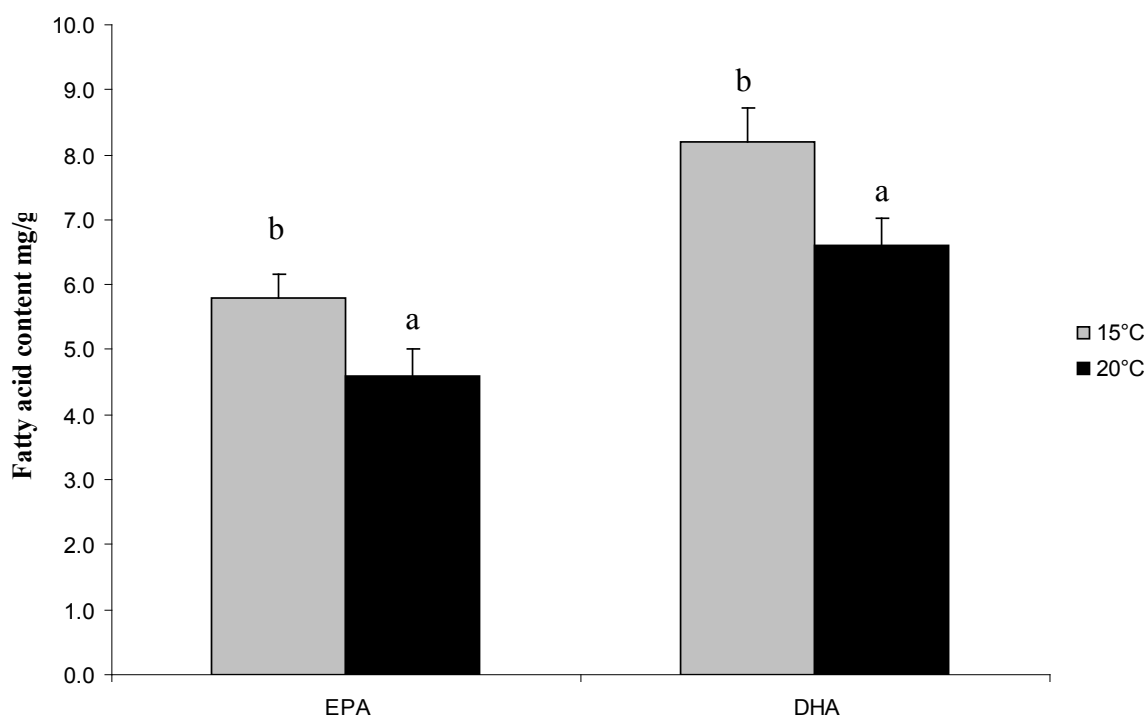


Figure 4.2: Effect of temperature on EPA and DHA content (mg/g, dry weight) in rainbow trout fillet after dietary shift from different dietary levels of PFAD for 77 days to a FO finishing diet for 28 days at 15°C and 20°C. Values are means \pm SEM of different dietary treatments grouped together within each temperature treatment; different letters represent significant differences in temperature, $P < 0.05$.

4.4.7 Regression analyses

The regression lines between predicted and observed %FA values (14:0, 16:0, 17:0, 18:0, Other SFA, 16:1n-7c, 18:1n-9c, 18:1n-7c, 20:1n-9, 20:1n-7c, 24:1n-9c, Other MUFA, 18:2n-6, 20:4n-6, Other n-6, 20:5n-3, 22:6n-3, Other n-3) for whole carcasses and fillet at both temperatures were highly significant (Fig 4.3). The regression lines had a strong degree of similarity with the line of equity, the lines crossed at the origin and slopes were close to 1. The same scenario was observed for selected C18 %FA values (18:0, 18:1n-9c, 18:1n-7c, 18:2n-6) (Fig 4.4), whereby regression lines were highly significant with a strong degree of similarity with the line of equity. These observations confirmed that the dilution model provides a good description of the changes in FA occurring in the fillet and whole carcass of rainbow

trout following dietary shift from different levels of PFAD (50% and 75%) to 100% FO at 15°C and 20°C.

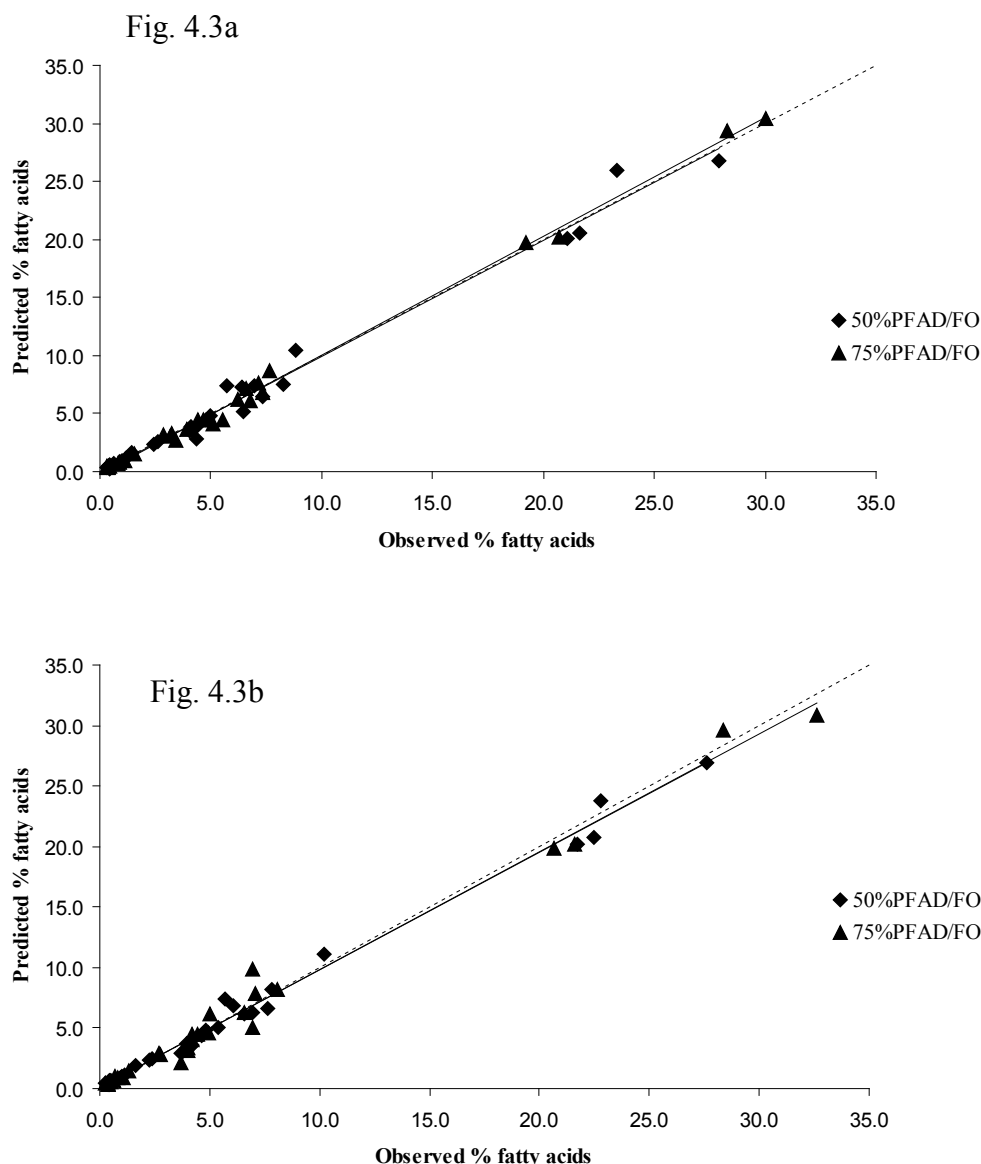


Figure 4.3: Total observed and predicted (from dilution model) fatty acid percentages in whole carcass and fillet of rainbow trout after dietary shift from 50% and 75% PFAD for 77 days to FO for 28 days at (a) 15°C (b) 20°C. 0%PFAD/FO – fish fed FO diet throughout, 50%PFAD/FO – fish fed 50% PFAD then on FO, 75%PFAD/FO – fish fed 75% PFAD then FO. (a) The regression lines (solid lines) are highly significant (50%PFAD/FO: $R^2 = 0.99$, $P < 0.001$; 75%PFAD/FO: $R^2 = 0.99$, $P < 0.001$), but not significantly different ($P > 0.05$) from the line of equality (dotted line). Line of equality shows a strong degree of similarity between observed values and those predicted from the dilution model. The regression equations are given by: Predicted = $-0.07 + 0.99$ Observed (50%PFAD/FO) and Predicted = $-0.18 + 1.02$ Observed (75%PFAD/FO). (b) The regression lines (solid lines) are highly significant (50%PFAD/FO: $R^2 = 0.99$, $P < 0.001$; 75%PFAD/FO: $R^2 = 0.99$, $P < 0.001$), but not significantly different ($P > 0.05$) from the line of equality (dotted line). Line of equality shows a strong degree of similarity between observed values and those predicted from the dilution model. The regression equations are given by: Predicted = $0.07 + 0.97$ Observed (50%PFAD/FO) and Predicted = $0.07 + 0.97$ Observed (75%PFAD/FO).

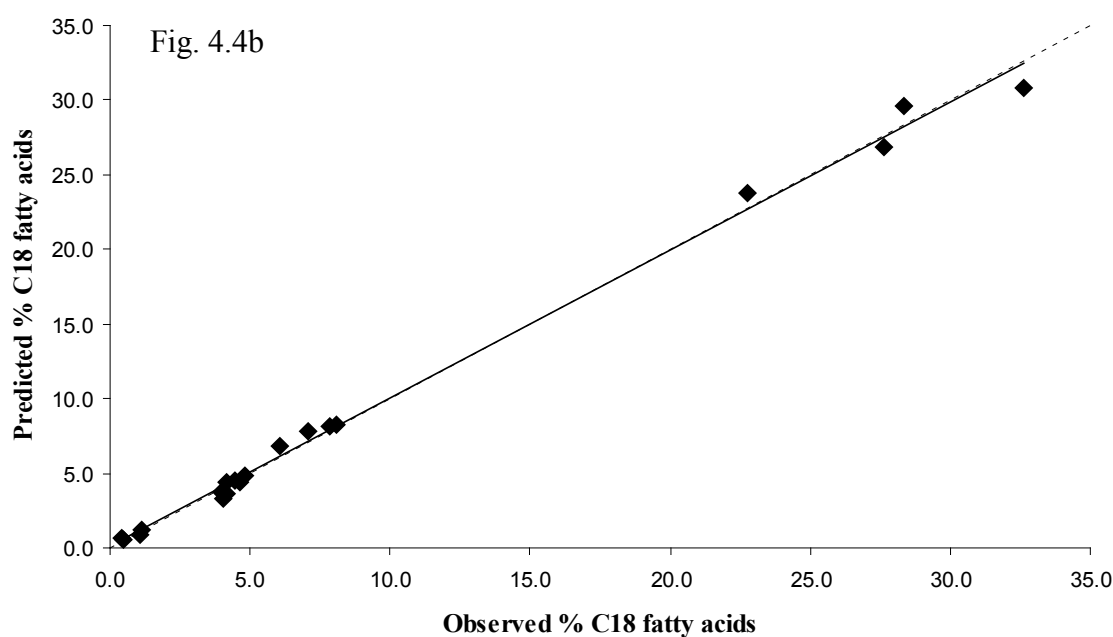
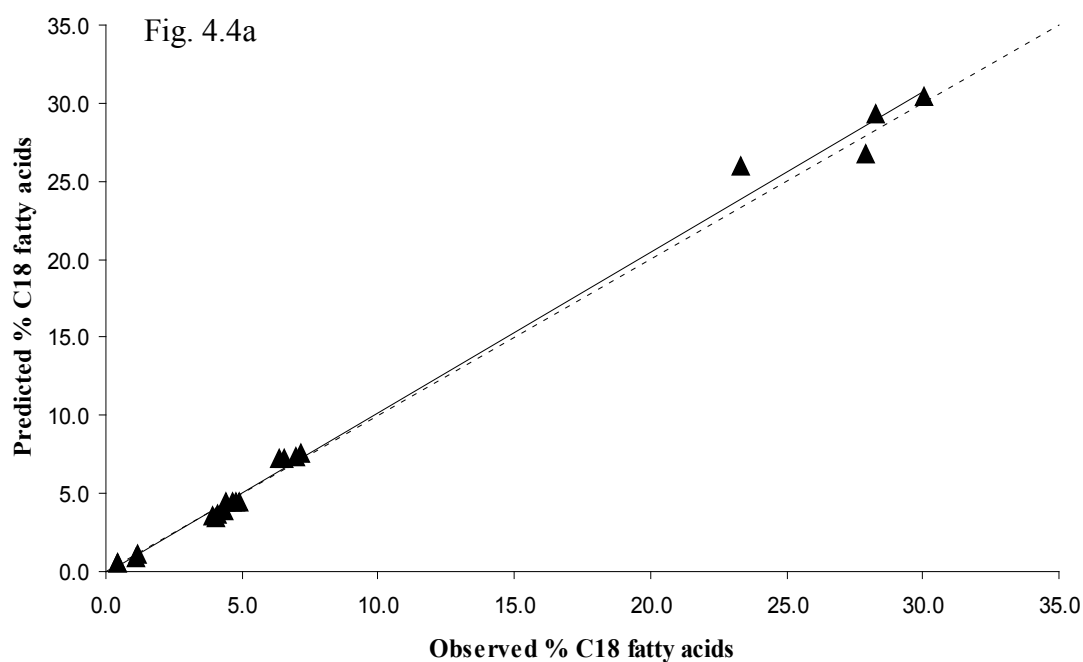


Figure 4.4: Total observed and predicted (from dilution model) fatty acid percentages of selected C18 FA in fillet and whole carcass of rainbow trout after dietary shift from different levels of PFAD for 77 days to FO for 28 days at (a) 15°C and (b) 20°C. (a) The regression line (solid line) is highly significant (PFAD/FO: $R^2 = 0.99$, $P < 0.001$), but not significantly different ($P > 0.05$) from the line of equality (dotted line). The line of equality shows a strong degree of similarity between observed values and those predicted from the dilution model. The regression equation is given by: Predicted = $-0.11 + 1.03$ Observed (PFAD/FO). (b) The regression line (solid line) is highly significant (PFAD/FO: $R^2 = 0.98$, $P < 0.001$), but not significantly different ($P > 0.05$) from the line of equality (dotted line). The line of equality shows a strong degree of similarity between observed values and those predicted from the dilution model. The regression equation is given by: Predicted = $0.09 + 0.99$ Observed (PFAD/FO).

4.5 DISCUSSION

High inclusion of palm oil at low water temperature reduced digestibility of SFA and subsequent energy availability; this is a major concern with the use of palm oil in salmonid aquafeeds (Ng et al., 2003; 2004a; 2007). PFAD is a by-product of the physical refining of crude palm oil and is characterised by high amounts of free fatty acids (~80%) and provides a relatively low-cost FA and it has been recently shown to improve the apparent digestibility of SFA in rainbow trout grown at optimum (15°C) and elevated (20°C) water temperatures (Ng et al., 2010). Despite improved apparent digestibility of SFA, the growth of fish especially that of 75%PFAD fish, was impaired at 15°C. The observed decreased growth is unclear given that the improved apparent digestibility of SFA will lead to increase energy availability. More research on the growth of rainbow trout with high dietary PFAD inclusion level should be undertaken. The primary aim of this study was to investigate the restoration of EPA and DHA in rainbow trout fed a FOFD at optimal and elevated temperatures. In order to not introduce another variable, fish were fed at fixed ration; consequently, growth was lower at 20°C due to higher energy demand as a result of the higher metabolic rates (Jobling, 1997; Katersky and Carter, 2007).

Modifications of FA in fillet and whole carcass of fish following dietary change from 50% and 75% PFAD to FO were generally not dependent on temperature. Feeding history during the grow-out period was the main determinant for the observed differences. At the end of the grow-out period, fillet and whole carcass %EPA and %DHA for fish fed on 50% and 75% PFAD was significantly lower than fish fed on 0% PFAD. The aim of the FOFD strategy is to restore levels of n-3 LC-PUFA especially in fish fillet after grow-out on alternative oils. At 50%

PFAD dietary inclusion level during the grow-out period, restoration of %EPA and %DHA was achieved to between 85% and 98% of that of fish fed FO throughout in whole carcass and fillet at both 15°C and 20°C. However with the higher PFAD inclusion level (75% PFAD) during the grow-out period, in the fillet, %EPA and %DHA was less (66% to 93%) restored at both 15°C and 20°C. These results indicate that at high PFAD inclusion levels (75%), a longer growth period on finishing FO diet is required to better restore fillet EPA and DHA levels. Extending the finishing period will obviously increase the use of FO which is a paradox, considering that the ultimate goal is to substitute FO. Even at the highest substitution level of FO (75%), over the whole culture period (105 days), more than 50% FO was used. The % values of DHA decreased for all treatments between the initial and final sampling for fillet and whole carcass. This observation is due to the lower DHA % composition of the FOFD as opposed to the 0%PFAD used during the grow-out period. These two diets were manufactured at different time points and different batches of FO were supplied and used and consequently variation in dietary FA % compositions arose.

FOFD diet strategies can be considered simply as a palliative solution to the problem of FO replacement in aquafeeds because complete restoration of optimal FA composition cannot be achieved and in attempting to do so a significant amount of FO is still required (Turchini et al., 2009). Where restoration of EPA and DHA was based solely on statistical interpretation, differences for EPA or DHA between treatments was either significant or non significant accompanied by a *P*-value. However, although there was no statistical difference in %EPA and %DHA in the fillet and whole carcass of fish fed 50% PFAD/FO and 0% PFAD/FO, fish in the latter treatments always had higher EPA and DHA percentage values. FA is routinely presented as %FA values in fish nutrition studies; however, if differences in lipid

content exist, it is important to also examine FA data as absolute concentration values (mg/g). We observed that there was no difference with diet in EPA and DHA in the fillet when absolute values (mg/g) were compared. Differences only existed between temperatures; fish grown at 15°C had higher EPA and DHA than fish at 20°C. The difference in total lipid content between treatments was largely responsible for the disparity between FA percentage and absolute values.

PFAD possesses important FA compositional attributes required during the grow-out period when applying a FOFD strategy, being rich in SFA and/or MUFA and low in C18 PUFA. This dietary FA profile allows preferential utilisation of SFA and MUFA while sparing EPA and DHA. These features form the basis of preferential FA metabolism as opposed to the dilution model where changes in FA in tissues after dietary change are the result of dilution of existing FA stores and deposition of new dietary FA in the tissues over time; the FA profiles gradually resemble those of fish fed the diet throughout. Despite the FA composition of PFAD preferentially favouring FA metabolism, we found no evidence of this occurring in fish fillet and whole carcass at both 15°C and 20°C. The dilution model provided a good description of FA changes occurring in fish fillet and whole carcass of rainbow trout after dietary change. Complete restoration of EPA and DHA was not achieved for fish fed on 75% PFAD/FO (% values), as the changes were mostly a function of time (finishing period) and level of FO substitution (grow-out period). Complete restoration of EPA and DHA in fish fillets after use of a finishing FO diet is rarely achieved, except for in a few studies (Izquierdo et al., 2005; Jobling et al., 2008; Turchini et al., 2006). It is very likely that complete restoration of EPA and DHA will occur in instances where lipid turnover/preferential FA metabolism is a contributory factor to FA changes in fillet. Regression analysis between observed and predicted

FA values of selected C18 FA, which are abundant in VO, provides good evidence for preferential FA metabolism. A slope significantly greater than one suggests that the changes in concentrations of C18 FA were more rapid than predicted by the model (Jobling et al., 2008). In our study, regression analysis of C18 FA did not show any evidence for preferential FA metabolism. PFAD has relatively low levels of n-6 PUFA especially LA compared to other VO such as rapeseed and soybean oils. In our study, the ratio of n-3: n-6 was lower in fish fillet and whole carcass of rainbow trout after growth on PFAD. After finishing with FO, the n-3: n-6 ratio was still lower for fish previously grown on PFAD, although n-3 LC-PUFA levels were increased. This is mainly due to LA being less readily metabolised, and thus, being retained for much longer periods in tissues (Bell et al., 2003a, b; Torstensen et al., 2004, 2005). This can be regarded as a constraint when using a finishing FO on fish previously fed VO, even when using PFAD which contains relatively low levels of LA. Nevertheless, the n-3: n-6 PUFA ratio in fillets of fish fed with palm oil-based diets are likely more favourable compared to fish fed with the more typical n-6 PUFA rich VO.

4.6 CONCLUSION

This study is the first to investigate the effectiveness of a FOFD strategy at two different temperatures for rainbow trout after growth on the novel palm based oil - PFAD. Feeding a 50% PFAD diet during the 77 day grow-out period followed by a FOFD for four weeks was sufficient for a large restoration (85% to 98%) of EPA and DHA %values in rainbow trout fillet and whole carcass. The 28 days FOFD period for fish previously fed 75% PFAD resulted in lower restoration (66% to 93%) of %EPA and %DHA in rainbow trout fillet. We showed that the dilution model was suitable to predict %FA modification occurring in the fillet and whole carcass of

rainbow trout after dietary change from different levels of PFAD to FO and there was no evidence for FA turnover or preferential FA metabolism. Although a large restoration of %EPA and %DHA was achieved for fish previously fed 50%PFAD at both optimal and elevated temperatures, the n-3: n-6 ratios were not fully restored to that of FO fish in the fillet and whole carcass.

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CHAPTER 5

Fillet n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid restoration by improved fish oil finishing diet strategy for Atlantic salmon (*Salmo salar* L.) smolts fed palm fatty acid distillate-based diet.

Adapted from Codabaccus, M.B., Bridle, A.R., Nichols, P.D., Carter, C.G., 2011. Fillet n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid restoration by improved fish oil finishing diet strategy for Atlantic salmon (*Salmo salar* L.) smolts fed palm fatty acid distillate-based diet. *In preparation*.

5.1 ABSTRACT

The absence of n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) in vegetable oils (VO), coupled with the low digestibility of saturated FA (SFA), restricts the use of crude palm oil for cold water fish species such as Atlantic salmon. Palm fatty acid distillate (PFAD) contains high amounts of free fatty acids and has the potential to improve the digestibility of SFA. Restoration of n-3 LC-PUFA by a fish oil finishing diet (FOFD) is principally a dilution of existing FA stores from previous diets, hence, reducing the lipid content in fish prior to feeding the FOFD may improve the efficiency of n-3 LC-PUFA restoration. This study had two main objectives: to determine 1. Whether feeding Atlantic salmon smolt a 75% PFAD diet (75PFAD) improves the digestibility of SFA and 2. Whether a 7 day food deprivation period (termed unfed, UF) after growth on 75PFAD leads to higher n-3 LC-PUFA restoration in the fish fillet when applying a FOFD. Comparison was made to fish fed a FO diet (FO) throughout at 15°C. Growth was not impaired for fish fed with 75PFAD for a period of 77 days. The apparent digestibility of SFA was significantly higher in 75PFAD compared to FO, while that of monounsaturated FA (MUFA) and PUFA were significantly lower for the 75PFAD fed fish. A 7 day food deprivation period significantly reduced the final lipid content in the fillet of 75PFAD/UF fish. The relative level (as % total FA) of n-3 LC-PUFA was higher in unfed fish compared to continuously fed fish after the 3 and 4 week FOFD periods, respectively. The results of this study suggest that PFAD is a potential FO substitute in aquafeeds for Atlantic salmon due to higher SFA digestibility and similar growth performance to FO fish. A short term food deprivation period prior to feeding a FOFD improves the efficiency of n-3 LC-PUFA restoration in the fillet.

5.2 INTRODUCTION

Substitution of fish oil (FO) in aquafeeds for salmonids has become inevitable due to the limited global supply of FO (Naylor et al., 2009; Turchini et al., 2009). Vegetable oils (VO) are common substitutes, but the main shortcoming with their use is the absence of n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) in such oils. Consequently, Atlantic salmon fed VO-based diets have lower n-3 LC-PUFA content compared to fish fed exclusively on FO-based diets (Rosenlund et al., 2001; Torstensen et al., 2004; Chapter 2 and Codabaccus et al., 2011). The low content of n-3 LC-PUFA in fish fed VO diet is generally not detrimental to fish growth and health (Miller et al., 2008; Turchini et al., 2009) as opposed to the decreased associated human health benefits gained in consuming fish (Seierstad et al., 2005). The human health promoting benefits of n-3 LC-PUFA have been increasingly acknowledged (Ruxton et al., 2007) and farmed Atlantic salmon can be a major source of these FA in the human diet (Miller et al., 2008). Since feeding fish a VO based diet reduces the n-3 LC-PUFA content in fish, feeding fish a FOFD for a period prior to harvest is an efficient way to restore n-3 LC-PUFA. It is generally accepted that the restoration of n-3 LC-PUFA is via dilution of existing carcass FA by feeding a n-3 LC-PUFA rich FO finishing diet (Jobling, 2004a). Feeding the FOFD for several months is generally required to restore n-3 LC-PUFA, particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), in Atlantic salmon (Bell et al., 2003, 2004; Torstensen et al., 2004, 2005). Consequently, considerable amounts of FO are still required in aquafeeds to fully restore n-3 LC-PUFA content in fish when using the FOFD strategy (Bell et al., 2004; Turchini et al., 2006; 2009). Preferential FA metabolism is another key process which can contribute to restoring n-3 LC-PUFA content when using a FOFD (Jobling et al., 2004b). In this respect, a diet rich in saturated fatty acids (SFA) and

monounsaturated fatty acids (MUFA) during the grow-out period has been proposed to be better suited because these FA groups are the preferred substrates for β -oxidation, thus sparing n-3 LC-PUFA when feeding the FOFD (Turchini et al., 2009). Palm fatty acid distillate (PFAD), a by-product of refining crude palm oil, is rich in free FA particularly SFA and MUFA (Bahurmiz and Ng, 2007), hence it has potential for use in a FOFD strategy. A major concern with high dietary SFA and MUFA is their lower apparent digestibility (AD) especially for cold water species such as Atlantic salmon (Turchini et al., 2009). PFAD predominantly contains free fatty acids (~80%) which have been shown to improve SFA digestibility in rainbow trout grown at optimal (15°C) and elevated (20°C) temperatures (Ng et al., 2010). To date there are no reports of the use of PFAD in diets for Atlantic salmon. Therefore it is of interest to examine the growth performance of Atlantic salmon fed a PFAD-based diet and to assess the restoration of n-3 LC-PUFA using a FOFD strategy. Atlantic salmon were fed a 100% FO or 75% PFAD diet for 77 days before being fed FOFD for 28 days. Furthermore and in anticipation that the restoration of n-3 LC-PUFA will follow mainly a dilution of existing FA stores, the effect of a short-term food deprivation (7 days) prior to feeding the FOFD (21 days) on n-3 LC-PUFA restoration was investigated. Due to logistic constraints with growing pre-harvest size fish in a recirculated system, Atlantic salmon smolts (~70g) were used and the investigation was principally conceptual.

5.3 METHODS

5.3.1 *Experimental diets*

Two isonitrogenous and isolipidic diets were formulated in which the added lipid source varied; one diet contained only FO and the other diet contained a ratio of 75% PFAD: 25% FO (75PFAD) (Table 5.1). PFAD was melted in a water bath and

thoroughly mixed with FO before the mixture was blended with dry ingredients. Diets were manufactured into 3 mm diameter pellets using a California Pellet Mill (CL-2, San Francisco, CA, USA), dried and stored at -5°C until use (Carter et al., 2003b). Yttrium oxide was added to the diets (1.0 g/kg) as an inert marker for measurement of apparent digestibility (AD) of fatty acids (Carter et al., 2003a).

Table 5.1: Ingredient and chemical composition of experimental diets

	Diet	
	FO	75PFAD
<i>Ingredient composition (g/kg)</i>		
Fishmeal ¹	300	300
Casein ²	50	50
Wheat gluten ³	100	100
Soybean meal ⁴	139	139
Fish oil ¹	200	50
Palm fatty acid distillate ⁵	0	150
Pre-gel starch ⁶	127	127
Vitamin mix ⁷	7	7
Mineral mix ⁸	7	7
Stay-C ⁹	6	6
Choline chloride ¹⁰	2	2
Sipernat ¹¹	40	40
CMC ¹⁰	10	10
Monobasic calcium phosphate ¹⁰	20	20
Yttrium oxide ¹⁰	1	1
<i>Chemical composition (g/kg DM)</i>		
Dry matter (g/kg)	916.6	919.9
Crude protein	356.2	354.3
Total lipid	235.1	233.6
Ash	106.2	104.1
Energy (MJ/kg)	19.8	19.7

¹Skretting Australia, Cambridge, Tasmania, Australia; ²MP Biomedicals Australasia Pty. Ltd., Seven Hills NSW, Australia; ³Starch Australasia, Lane Cove, NSW, Australia; ⁴Hamlet Protein A/S, Horstens, Denmark; ⁵Wilmar Edible Oils Ltd., Penang, Malaysia; ⁶Penford Limited, Lane Cove, NSW, Australia; ⁷Vitamin mix (ASV4) (Carter et al. 2003a); ⁸Mineral mix (TMV4) (Carter et al. 2003a); ⁹L-Ascorbyl-2-polyphosphate (Roche Vitamins Australia, Frenchs Forest, NSW, Australia); ¹⁰Sigma-Aldrich, Castle Hill, NSW, Australia; ¹¹Degussa, Frankfurt, Germany. FO; fish oil diet; 75PFAD, 75% palm fatty acid diet.

5.3.2 Growth experiment

The experiment was conducted at the University of Tasmania (Launceston, Tasmania, Australia) in accordance with the University of Tasmania Animal Ethics

guidelines (Investigation A0009731). Atlantic salmon (*Salmo salar* L.) smolts, of average weight ~ 70 g obtained from Wayatinah Salmon Hatchery (SALTAS, Tasmania, Australia), were acclimated in seawater for a period of 14 days before the experiment. Fish were fed a commercial diet (Skretting, Tasmania, Australia) during the acclimation period. After acclimation, fish were randomly distributed in six 300 L fibreglass tanks at a stocking density of 35 fish/tank. Rearing tanks were connected to a partial recirculating system equipped with a protein skimmer and physical, UV and biological filters (Carter and Hauler, 2000). At the start of the experiment, fish were anaesthetized (50 mg/L, benzocaine), weighed and fork length measured. Six fish were euthanized (100 mg/L) and fillet was dissected and stored at -20 °C for measurement of initial lipid content and FA composition. Fish were fed one of the two experimental diets (3 replicate tanks/diet) at a fixed ration of 1.5% body weight/d (BW/d) in two equal rations and water temperature was kept constant at 15 °C. Every 14 days fish were bulk weighed to readjust the feed ration.

After 42 days, fish from each tank were randomly removed for faecal collection until there were 20 fish remaining in each tank. Three hours after the last feeding, fish from each tank were anaesthetised (50 mg/L, benzocaine) and faecal samples collected from the hind gut region by gently squeezing the ventral abdominal area (Percival et al., 2001). Faecal samples were pooled by tank and stored at -20 °C prior to analysis of FA composition and yttrium oxide. After faecal stripping, fish were euthanized (100 mg/L, benzocaine). Remaining fish were grown for a further 35 days on the two experimental diets. At the end of the 77 day growth period, fish were bulk weighed. Two fish per tank were weighed, their fork length measured and viscera and fillet were dissected and stored at -20 °C prior to analysis of lipid content and FA composition. Remaining fish per tank (~16) were pooled per treatment (~49)

and fish fed on 75PFAD diet were randomly allocated to four tanks (12 fish/tank). Fish fed on FO diet were randomly allocated to two tanks (12 fish/tank). All fish were then fed at 1.5% BW on FO diet except for fish in two tanks previously fed 75PFAD diet which were unfed for 7 days. After 7 days of food deprivation, six fish per treatment [fish fed FO throughout (FO), fish fed 75PFAD then FO (75PFAD/FO), fish fed 75PFAD then unfed (75PFAD/UF)] were dissected for viscera and fillet and stored at -20°C prior to analysis of total lipid content and FA composition. All treatments were then fed to satiation on FO diet for a further 21 days. At the end of the FOFD period, six fish per treatment [FO, 75PFAD/FO, fish fed 75PFAD then unfed then FO (75PFAD/UF/FO)] were dissected to obtain viscera and fillet and stored at -20°C prior to analysis of lipid content and FA composition. An illustration of the experimental design is presented in Figure 5.1.

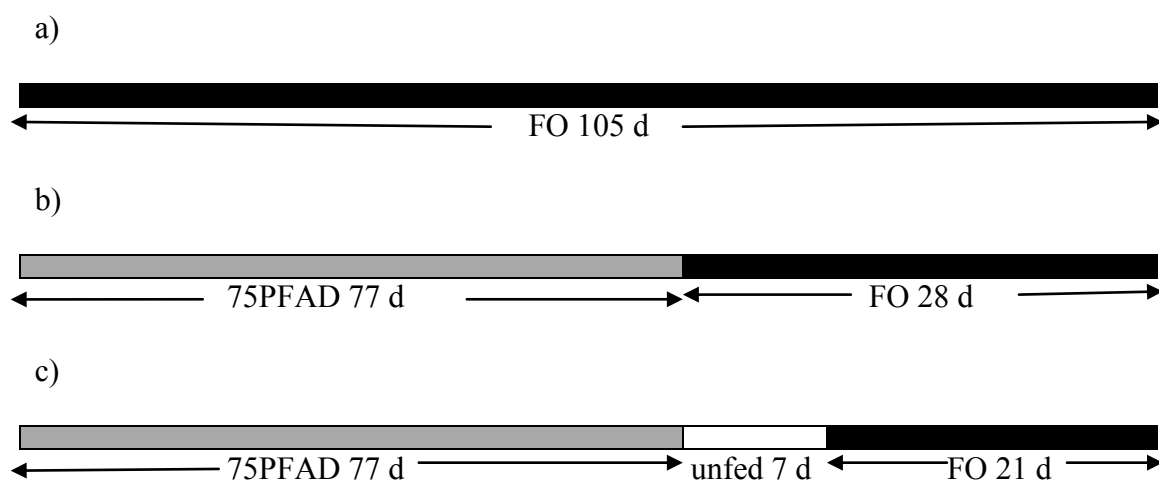


Figure 5.1: Schematic illustration of treatments. d denotes period in days. a) Fish fed for 105 days with fish oil diet throughout (FO). b) Fish fed for 77 days with 75PFAD, followed by 28 days with FOFD (75PFAD/FO). c) Fish fed for 77 days with 75PFAD, followed by 7 days of food deprivation, then fed with FOFD for 21 days (75% PFAD/UF/FO).

5.3.3 Apparent digestibility

Faecal samples were freeze-dried prior to chemical analysis. AD was calculated using the formula $AD (\%) = 100 - [100 (Y_{\text{diet}}/Y_{\text{faeces}}) * (FA_{\text{faeces}}/FA_{\text{diet}})]$ where Y is the % of yttrium oxide and FA is the % of particular fatty acids (Maynard & Loosli, 1969).

5.3.4 Chemical analysis

Standard methods were used to determine dry matter (DM) (freeze dry to constant weight then drying at 135°C for 2 h) (AOAC, 1995) of experimental diets; total lipid (Bligh and Dyer, 1959); nitrogen (Kjedhal using selenium catalyst; crude protein was calculated as N x 6.25); energy (bomb calorimeter, Gallenkamp Autobomb, calibrated with benzoic acid) and ash by combustion at 600°C for 2 h (AOAC, 1995). Apart from DM, freeze dried samples were used for all analyses and corrected for DM.

5.3.5 Lipid extraction, lipid class and fatty acid analyses

Diets, fillet, viscera and faecal samples were freeze dried and extracted overnight using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single phase extraction using $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (1:2:0.8, v/v/v), followed by phase separation to yield a total lipid extract (TLE).

An aliquot of the TLE was trans-methylated in methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 2 h at 100°C. After addition of MilliQ water (1 ml), the mixture was extracted with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). Samples with an internal injection standard (19:0 FAME) added were analysed by gas chromatography (GC) using an Agilent Technologies

7890B GC (Palo Alto, California, USA) equipped with an Equity™-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 µm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683 B Series auto sampler. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 120°C. After injection, oven temperature was raised to 270°C at 10°C/min and finally to 310°C at 5°C/min. Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, California, USA). GC results are typically subject to an error of up to ± 5% of individual component area.

Individual components were identified by mass spectral data and by comparing retention time data with authentic and laboratory standards. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, Texas, USA). The GC was equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.). Helium was used as carrier gas, with operating conditions previously described (Miller et al., 2006).

5.3.6 Biometrics and statistical analysis

Specific growth rate (SGR) was calculated as $SGR (\%/d) = 100 * (\ln W_f / \ln W_i) / d$ where W_f and W_i are the final and initial weights (g) and d is the number of days of the experiment. Feed consumption (FC) was calculated as the total average amount of dry feed (g) consumed per tank over the number of days of the experiment. The feed efficiency ratio was calculated as $FER (g/g) = \text{total weight gain (g)} / FC (g)$. Condition factor (K) was calculated as $K (\%) = 100 * (W / FL^3)$, where FL was the fork length (cm). The viscera somatic index (VSI) was calculated as $VSI = 100 *$

(weight of viscera/W). Values are reported as mean \pm standard error of the mean (SEM). Normality and homogeneity of variance were confirmed and percentage data were arcsine transformed prior to analysis. Samples from individual fish ($n=6$) were compared between treatment means for FA composition and growth performance was by independent samples t-test and one way ANOVA followed by multiple comparisons using Tukey-Kramer HSD wherever applicable. Significance was accepted at probabilities $P < 0.05$.

The dilution model is expressed as follows (Robin et al., 2003):

$$P_t = P_r + (P_i - P_r)/(Q_t - Q_i)$$

where P_t is defined as the percentage of FA in the fillet and viscera of fish fed 75% PFAD/FO at time t , P_i is the initial percentage of FA in the fillet of fish previously fed 75% PFAD and P_r is the percentage of FA in the fillet and viscera of fish fed FO throughout at time t . Q_i is the initial total amount of lipid present and Q_t is the amount present at time t in the fillet and viscera of fish fed 75% PFAD/FO. A regression analysis between predicted (from the dilution model) and observed FA values was performed and individual regression lines were compared to the line of equity (Jobling, 2004b). Analysis of covariance (ANCOVA) was used to compare slopes and intercept of regression lines to the line of equity. Statistical analysis was performed using SPSS for Windows version 16.0.

5.4 RESULTS

5.4.1 Growth

There was no significant difference in SGR, FER and body indices between FO and 75PFAD fish during the grow-out period (Table 5.2). Unfed fish (75PAFD/UF) had lower viscera weight, K and VSI than FO and 75PFAD/FO fish

during the first 7 days of the FOFD period. Similarly, 75PAFD/UF fish had lower viscera weight, K and VSI when compared to 75PFAD fish. There was no significant difference for all treatments in SGR, FER and body indices except for lower VSI in 75PFAD/FO fish at the end of the 28 days FOFD period.

Table 5.2: Growth, feed efficiency and body indices of Atlantic salmon fed either FO or 75PFAD during the grow-out period for 77 days followed by either 28 days FOFD or 7 days food deprivation followed by 21 days FOFD

	Initial weight (g)			Final weight (g)			SGR (%W)			FER (g/g)			K			Fillet weight (g)			Viscera weight (g)			VSI (%)		
Grow-out (day 77)																								
FO	71.5	±	1.9	147.9	±	4.1	0.97	±	0.0	1.01	±	0.0	1.30	±	0.0	48.3	±	6.3	17.3	±	1.6	9.7	±	0.1
75PFAD	73.5	±	1.0	152.8	±	3.3	0.98	±	0.0	1.02	±	0.0	1.32	±	0.0	40.8	±	6.9	15.4	±	2.2	9.9	±	0.3
Finishing (day 84)																								
FO													1.34	±	0.0 ^a	41.1	±	3.3	16.6	±	0.9 ^a	10.6	±	0.4 ^a
75 PFAD/FO													1.38	±	0.0 ^a	40.8	±	3.5	16.5	±	0.9 ^a	10.8	±	0.5 ^a
75PFAD/UF													1.21	±	0.0 ^{b*}	38.4	±	3.8	12.5	±	1.0 ^{b*}	8.7	±	0.3 ^{b*}
Finishing (day 105)																								
FO	154.3	±	2.4	202.8	±	2.3	0.98	±	0.0	1.02	±	0.0	1.33	±	0.0	54.2	±	4.2	21.0	±	1.0	10.8	±	0.4 ^{ab}
75PFAD/FO	157.6	±	4.3	208.0	±	6.4	0.99	±	0.0	1.01	±	0.0	1.36	±	0.0	54.3	±	3.8	20.2	±	0.7	10.0	±	0.3 ^b
75PFAD/UF/FO	154.4	±	4.1	193.5	±	10.1	1.07	±	0.1	1.00	±	0.0	1.37	±	0.0	57.2	±	4.6	23.0	±	1.8	11.3	±	0.3 ^a

Values are means ± SEM, $n = 3$ for initial weight, final weight, SGR and FER, $n = 6$ for fillet weight, viscera weight, K and VSI. Means in a column belonging to different feeding periods sharing different superscript letters were significantly different ($P < 0.05$). An (*) represents significant difference ($P < 0.05$) in K and VSI between fish fed 75PFAD and fish fed 75PFAD then unfed for 7 days. FO, fish fed FO; 75PFAD, fish fed 75PFAD; 75PFAD/FO, fish fed 75PFAD then fed FOFD; 75PFAD/UF, fish fed 75PFAD then unfed for 7 days; 75PFAD/UF/FO, fish fed 75PFAD then unfed for 7 days then fed FOFD for 21 days.

5.4.2 Fatty acid analyses – diet

Total SFA and total MUFA were higher in 75PFAD than in the FO diet due to two-fold higher 16:0 and a around three-fold difference in 18:1n-9c (Table 5.3). There was higher PUFA in FO compared to 75PFAD, particularly total n-3 and total n-3 LC-PUFA. The n-3: n-6 ratio was higher in the FO diet compared to 75PFAD.

Table 5.3: Fatty acid composition (% total fatty acids) of experimental diets

FA	Diet		
	FO		
			75PFAD
14:0	3.3	± 0.1 ^a	1.5 ± 0.1 ^b
16:0	19.9	± 0.0 ^b	38.2 ± 0.1 ^a
17:0	0.5	± 0.0	0.3 ± 0.0
18:0	4.1	± 0.0	4.5 ± 0.0
Other SFA ¹	1.3	± 0.0 ^a	0.4 ± 0.1 ^b
16:1n-7c	9.2	± 0.0 ^a	2.6 ± 0.0 ^b
18:1n-7c	4.0	± 0.0 ^a	1.9 ± 0.0 ^b
18:1n-9c	11.0	± 0.1 ^b	29.4 ± 0.1 ^a
20:1n-7c	0.4	± 0.0 ^a	0.1 ± 0.0 ^b
20:1n-9	0.9	± 0.0 ^a	0.4 ± 0.0 ^b
22:1n-11c	0.6	± 0.0 ^a	0.3 ± 0.0 ^b
24:1n-9c	0.4	± 0.0 ^a	0.1 ± 0.0 ^b
Other MUFA ²	1.2	± 0.0 ^a	0.3 ± 0.2 ^b
18:2n-6	3.6	± 0.0 ^b	8.8 ± 0.0 ^a
20:4n-6	0.8	± 0.0 ^a	0.3 ± 0.0 ^b
Other n-6	0.6	± 0.0 ^a	0.2 ± 0.0 ^b
18:3n-3	0.7	± 0.0 ^a	0.4 ± 0.0 ^b
18:4n-3	2.6	± 0.0 ^a	0.6 ± 0.0 ^b
20:5n-3	18.0	± 0.0 ^a	4.8 ± 0.1 ^b
22:5n-3	2.1	± 0.0 ^a	0.5 ± 0.0 ^b
22:6n-3	9.0	± 0.0 ^a	2.8 ± 0.0 ^b
Other n-3	1.7	± 0.0 ^a	0.5 ± 0.0 ^b
Other PUFA ³	4.2	± 0.0 ^a	1.1 ± 0.0 ^b
Total SFA	29.1	± 0.1 ^b	45.0 ± 0.0 ^a
Total MUFA	27.7	± 0.1 ^b	35.1 ± 0.1 ^a
Total PUFA	43.2	± 0.0 ^a	19.9 ± 0.1 ^b
Total n-3	34.0	± 0.0 ^a	9.5 ± 0.2 ^b
Total n-3 LC-PUFA	30.8	± 0.0 ^a	8.6 ± 0.0 ^b
Total n-6	5.1	± 0.0 ^b	9.2 ± 0.1 ^a
n-3: n-6	6.7	± 0.1 ^a	1.0 ± 0.1 ^b

Values are means ± SEM, $n = 3$. Means in a row sharing different superscript letters were significantly different ($P < 0.05$). FO; fish oil diet; 75PFAD, 75% palm fatty acid diet. ¹Saturated fatty acids; ²Monounsaturated fatty acids; ³Polyunsaturated fatty acids.

5.4.2 Apparent digestibility (AD)

AD generally decreased with increasing FA chain length and the AD increased with increasing degree of FA unsaturation (Table 5.4). AD of total PUFA was highest followed by total MUFA and was lowest for total SFA. AD of all SFA measured was

higher for 75PFAD compared to FO. AD of total MUFA and total PUFA was lower for 75PFAD than FO.

Table 5.4: Fatty acid apparent digestibility (%) of experimental diets fed to Atlantic salmon

FA	Diet	
	FO	75PFAD
14:0	83.6 ± 1.3 ^b	92.5 ± 1.7 ^a
16:0	67.5 ± 1.7 ^b	78.5 ± 1.3 ^a
17:0	60.0 ± 1.6 ^b	81.2 ± 1.8 ^a
18:0	52.5 ± 2.2 ^b	75.1 ± 1.4 ^a
16:1n-7c	96.9 ± 0.1	96.5 ± 0.4
18:1n-7c	91.5 ± 0.2	91.1 ± 0.6
18:1n-9c	93.4 ± 0.2 ^a	90.6 ± 0.6 ^b
20:1n-7c	82.1 ± 0.5	92.3 ± 3.8
20:1n-9	89.8 ± 0.2	91.8 ± 1.3
22:1n-11c	86.0 ± 0.9	90.0 ± 2.7
24:1n-9c	64.8 ± 1.0 ^b	75.3 ± 2.8 ^a
18:2n-6	91.8 ± 0.1	93.2 ± 0.5
20:4n-6	98.9 ± 1.1	100.0 ± 0.0
18:3n-3	96.6 ± 2.0	96.7 ± 1.7
18:4n-3	99.1 ± 0.1	100.0 ± 0.0
20:5n-3	98.7 ± 0.1	97.7 ± 0.4
22:5n-3	97.5 ± 0.0	97.4 ± 1.5
22:6n-3	96.0 ± 0.1	95.0 ± 0.5
Total SFA	67.3 ± 1.7 ^b	78.5 ± 1.3 ^a
Total MUFA	93.2 ± 0.2 ^a	90.9 ± 0.6 ^b
Total PUFA	97.6 ± 0.1 ^a	95.6 ± 0.4 ^b

Values are means ± SEM, $n = 3$. Means in a row sharing different superscript letters were significantly different ($P < 0.05$). FO; fish oil diet; 75PFAD, 75% palm fatty acid diet.

5.4.4 Lipid and fatty acid analyses – fillet

Fish fed FO diet throughout had the highest relative levels (as % of total FA) of EPA, DHA, total n-3, n-3 LC-PUFA and n-3: n-6 ratio at all three sampling points except for the 75PFAD/UF fish where DHA was not significantly different to the FO fish (Table 5.5). Total SFA (as % of total FA) was not significantly different between treatments after the 77 days grow-out period. Total MUFA level was higher for 75PFAD fish compared to FO fish and total PUFA of FO fish was higher than that of 75PFAD fish after the grow-out period. EPA, DHA and n-3 LC-PUFA of 75PFAD fish after grow-out were 51%, 25% and 36% respectively lower than that of FO fish.

After 7 days feeding the FOFD, 75PFAD/FO fish had lower relative levels of DHA, total n-3 and n-3 LC PUFA than 75PFAD/UF fish (unfed for 7 days). Similarly, compared to 75 PFAD/UF fish, 75PFAD fish (grow-out) had lower relative levels of DHA, total n-3 and n-3 LC-PUFA. The lipid content (DM) of 75PFAD/UF fish was lower than 75PFAD fish.

At the end of the 21 days and 28 days FOFD periods for 75PFAD/UF/FO fish and 75PFAD/FO fish respectively, 75PFAD/UF/FO fish had higher relative levels of EPA, total PUFA, total n-3 and n-3 LC-PUFA than 75PFAD/FO fish. The relative levels of EPA, DHA and n-3 LC-PUFA in the fillet of PFAD/FO fish was restored to 62%, 80% and 72% respectively, of that of FO fish after 28 days of the FOFD period. EPA, DHA and n-3 LC-PUFA % composition in the fillet of 75PFAD/UF/FO fish was restored to 75%, 86% and 81% respectively, of that of FO fish after 21 days FOFD period.

FO fish had higher absolute amounts of n-3 LC-PUFA after 77 days growth period compared to 75PFAD fish (Fig 5.2). In contrast to the relative percentage FA levels, there was no difference in absolute amounts of n-3 LC-PUFA between 75PFAD/UF fish and initial fish (75PFAD). Similarly, there was no difference in absolute amounts of n-3 LC-PUFA between 75PFAD/UF fish and 75PFAD/FO fish after 7 days food deprivation and 7 days FOFD respectively. FO fish had higher amounts of n-3 LC-PUFA at the end of 28 days FOFD compared to 75PFAD/FO fish and 75PFAD/UF/FO fish. Similar to results comparing the relative levels of FA, absolute amounts of n-3 LC-PUFA in 75PFAD fish after grow-out were 37% lower than that of FO fish and restoration of n-3 LC-PUFA content in 75PFAD/FO fish and

75PFAD/UF/FO fish after 28 days and 21 days FOFD respectively, was 71% and 80% respectively.

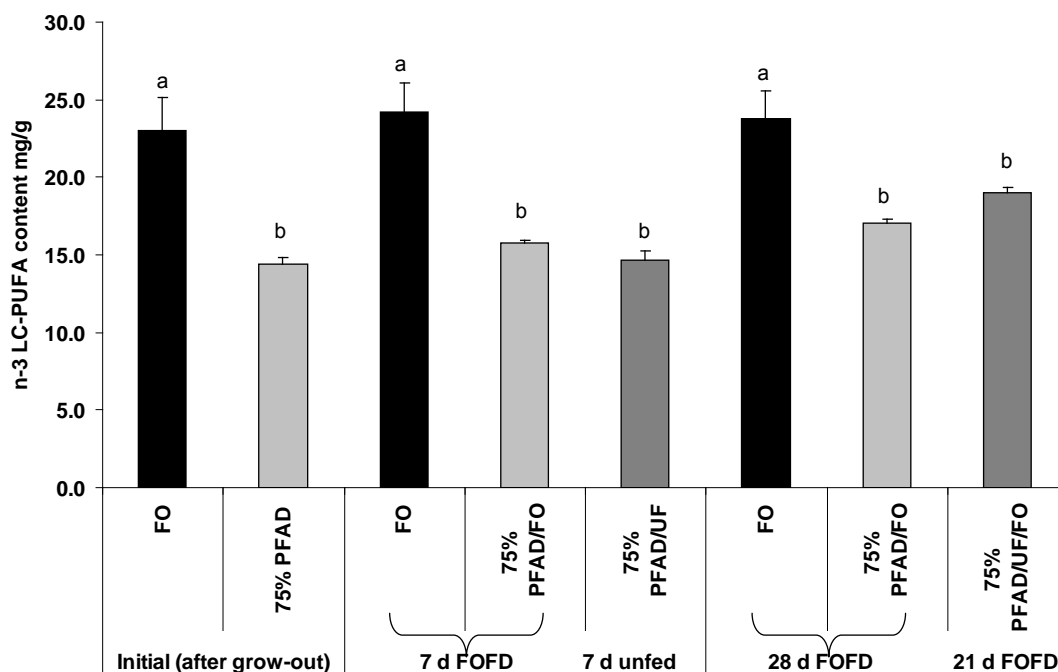


Figure 5.2: n-3 LC-PUFA content (mg/g) in the fillet of Atlantic salmon fed 75PFAD diet and FO diet for 77 days followed by either 28 days feeding on FOFD (75PFAD/FO fish) or 7 days food deprivation and 21 days feeding on FOFD (75PFAD/UF/FO). d denotes periods in days. Values are means \pm SEM, $n = 6$. Different letters represent significant differences ($P < 0.05$) between treatments at same sampling periods.

5.4.5 Lipid and fatty acid analyses – viscera

Fish fed FO throughout had the highest relative levels of EPA, DHA, total n-3, n-3 LC-PUFA and n-3: n-6 ratio at all three sampling points (Table 5.6). Total SFA was not different between treatments after the grow-out period. Total MUFA levels were higher for 75PFAD fish compared to FO fish and total PUFA of FO fish was higher than that of 75PFAD fish after the grow-out period. EPA, DHA and n-3 LC-PUFA of 75PFAD fish after grow-out were 62%, 34% and 47% lower respectively than that of FO fish. After 7 days feeding the FOFD, 75PFAD/FO fish had higher relative levels of DHA, total n-3 and n-3 LC PUFA than 75PFAD/UF fish (unfed for 7 days). In contrast to the fillet, there was no significant difference for the viscera in

the relative levels of any FA inclusive of total n-3, total PUFA and n-3 LC-PUFA between 75PFAD fish (after grow-out) and 7 days unfed fish (75PFAD/UF). There was also no significant difference in lipid content of viscera between 75PFAD/FO fish and 75PFAD/UF fish. These findings indicate that 7 days of food deprivation did not affect the FA profile and lipid content of the viscera. At the end of 28 days and 21 days FOFD period for 75PFAD/FO fish and 75PFAD/UF/FO fish respectively, there was no significant difference in the FA profile of the viscera. Relative (% of total FA) levels of EPA, DHA and n-3 LC-PUFA in the viscera of 75PFAD/FO fish and 75PFAD/UF/FO fish after 28 days and 21 days FOFD respectively, were restored to 56%, 71% and 66% of that of FO fish.

Table 5.5: Fatty acid composition (% of total FA) and lipid content (mg/g) of the fillet of Atlantic salmon fed either FO or 75PFAD during the grow-out period followed by either FOFD periods of 7 days and 28 days or 7 days food deprivation and 21 days FOFD periods

FA	Grow-out (day 77)					FOFD or unfed (day 84)					FOFD (day 105)						
	FO		75PFAD			FO		75PFAD/FO			75PFAD/UF		FO		75PFAD/FO		75PFAD/UF/FO
14:0	2.6	± 0.2 ^a	1.6	± 0.2 ^b		1.8	± 0.1	1.7	± 0.1	1.5	± 0.1	2.7	± 0.2 ^a	2.0	± 0.1 ^b	1.5	± 0.0 ^b
16:0	18.1	± 0.3 ^b	19.6	± 0.4 ^a		18.4	± 0.1	19.2	± 0.3	19.5	± 0.4	17.2	± 0.6 ^b	19.1	± 0.4 ^a	17.9	± 0.0 ^{ab}
17:0	0.4	± 0.0 ^a	0.3	± 0.0 ^b		0.4	± 0.0	0.2	± 0.1	0.3	± 0.0	0.4	± 0.0 ^a	0.3	± 0.0 ^b	0.3	± 0.0 ^b
18:0	4.5	± 0.1	4.5	± 0.1		4.8	± 0.0 ^a	4.6	± 0.0 ^b	4.6	± 0.1 ^b	4.5	± 0.2	4.5	± 0.0	4.4	± 0.0
16:1n-7c	7.8	± 0.2 ^a	4.6	± 0.1 ^{b*}		7.5	± 0.0 ^a	5.3	± 0.1 ^b	4.1	± 0.1 ^c	7.9	± 0.4 ^a	5.9	± 0.2 ^b	5.7	± 0.2 ^b
18:1n-7c	4.4	± 0.0 ^a	3.2	± 0.0 ^b		4.4	± 0.0 ^a	3.4	± 0.0 ^b	3.0	± 0.1 ^c	4.4	± 0.0 ^a	3.7	± 0.1 ^b	3.7	± 0.1 ^b
18:1n-9c	13.6	± 0.2 ^b	28.2	± 1.0 ^a		13.9	± 0.2 ^b	26.8	± 0.2 ^a	25.8	± 0.6 ^a	13.5	± 0.6 ^b	24.0	± 0.8 ^a	22.9	± 0.1 ^a
20:1n-7c	0.3	± 0.0 ^a	0.2	± 0.0 ^b		0.3	± 0.0	0.2	± 0.0	0.2	± 0.0	0.3	± 0.0	0.3	± 0.0	0.3	± 0.0
20:1n-9	1.6	± 0.1 ^b	1.9	± 0.1 ^a		1.5	± 0.0 ^b	1.9	± 0.1 ^a	1.8	± 0.1 ^{ab}	1.7	± 0.1	1.8	± 0.1	1.8	± 0.1
22:1n-11c	0.6	± 0.1	0.6	± 0.0		0.8	± 0.0 ^a	0.6	± 0.1 ^{ab}	0.5	± 0.0 ^b	0.6	± 0.0	0.5	± 0.0	0.6	± 0.1
24:1n-9c	0.4	± 0.0	0.4	± 0.0		0.5	± 0.0	0.4	± 0.0	0.4	± 0.0	0.4	± 0.0	0.3	± 0.0	0.3	± 0.0
18:2n-6	3.7	± 0.2 ^b	7.1	± 0.2 ^a		3.6	± 0.0 ^b	6.6	± 0.0 ^a	6.6	± 0.3 ^a	4.2	± 0.6 ^b	6.5	± 0.4 ^a	6.4	± 0.1 ^a
20:4n-6	0.8	± 0.0 ^a	0.6	± 0.0 ^{b*}		0.8	± 0.0 ^a	0.6	± 0.0 ^b	0.8	± 0.0 ^a	0.7	± 0.0	0.6	± 0.0	0.7	± 0.1
18:3n-3	0.6	± 0.1	0.5	± 0.1		0.6	± 0.0 ^a	0.4	± 0.0 ^b	0.4	± 0.0 ^b	0.8	± 0.2	0.5	± 0.1	0.5	± 0.0
18:4n-3	2.3	± 0.0 ^a	1.2	± 0.0 ^b		2.2	± 0.1 ^a	1.3	± 0.2 ^b	1.1	± 0.0 ^b	2.2	± 0.1 ^a	1.7	± 0.1 ^b	1.7	± 0.1 ^b
20:5n-3	10.8	± 0.2 ^a	5.3	± 0.3 ^b		10.8	± 0.2 ^a	5.8	± 0.0	6.1	± 0.2	10.6	± 0.4 ^a	6.6	± 0.1 ^c	7.9	± 0.2 ^b
22:5n-3	4.1	± 0.1 ^a	2.4	± 0.1 ^b		4.0	± 0.0 ^a	2.6	± 0.0 ^b	2.5	± 0.0 ^b	4.0	± 0.3 ^a	2.8	± 0.1 ^b	3.1	± 0.1 ^b
22:6n-3	15.9	± 0.7 ^a	11.9	± 0.7 ^{b*}		16.5	± 0.1 ^a	12.3	± 0.1 ^b	15.3	± 0.7 ^a	15.9	± 0.4 ^a	12.7	± 0.3 ^b	13.7	± 0.5 ^b
Total SFA	26.4	± 0.6	26.7	± 0.5		26.3	± 0.1	26.4	± 0.2	26.4	± 0.6	25.9	± 0.7	26.7	± 0.8	25.1	± 0.1
Total MUFA	29.7	± 0.4 ^b	39.9	± 0.8 ^a		29.8	± 0.3 ^c	39.4	± 0.2 ^a	36.7	± 1.0 ^b	29.5	± 0.5 ^b	37.1	± 0.7 ^a	36.0	± 0.5 ^a
Total PUFA	43.9	± 0.8 ^a	33.4	± 1.3 ^b		43.9	± 0.2 ^a	34.2	± 0.3 ^c	36.8	± 0.4 ^b	44.5	± 0.3 ^a	36.2	± 0.1 ^c	38.9	± 0.6 ^b
Total n-3	36.2	± 0.9 ^a	22.8	± 1.3 ^{b*}		36.5	± 0.1 ^a	24.1	± 0.2 ^c	26.7	± 0.7 ^b	36.1	± 0.4 ^a	26.1	± 0.3 ^c	28.9	± 0.5 ^b
Total n-6	5.4	± 0.3 ^b	9.4	± 0.2 ^a		5.2	± 0.0 ^b	8.6	± 0.1 ^a	8.9	± 0.3 ^a	5.9	± 0.7 ^b	8.4	± 0.4 ^a	8.3	± 0.2 ^a
n-3 LC-PUFA	33.2	± 1.0 ^a	21.1	± 1.2 ^{b*}		33.7	± 0.2 ^a	22.4	± 0.1 ^c	25.2	± 0.8 ^b	33.0	± 0.5 ^a	23.9	± 0.3 ^c	26.6	± 0.6 ^b
n-3: n-6	6.8	± 0.4 ^a	2.4	± 0.2 ^b		7.0	± 0.0 ^a	2.8	± 0.1 ^b	3.0	± 0.1 ^b	6.2	± 0.7 ^a	3.1	± 0.2 ^b	3.5	± 0.0 ^b
Lipid content (mg/g)																	
Dry	90.4	± 9.2	90.1	± 1.4*		88.8	± 6.7	89.9	± 2.2	78.5	± 2.8	105.2	± 3.6	102.7	± 4.9	106.7	± 1.8
Wet	28.1	± 3.4	25.3	± 0.5		25.7	± 1.4	26.5	± 1.0	22.0	± 1.1	31.2	± 1.1	31.9	± 1.1	31.9	± 1.1

Values are means ± SEM, $n = 6$. Means in a row belonging to different feeding periods sharing different superscript letters were significantly different ($P < 0.05$). An (*) represents significant difference ($P < 0.05$) in % FA and lipid content between fish fed 75 PFAD and fish fed 75PFAD then unfed for 7 days. FO, fish fed FO; 75PFAD, fish fed 75PFAD; 75PFAD/FO, fish fed 75PFAD then fed FOFD; 75PFAD/UF, fish fed 75PFAD then unfed for 7 days; 75PFAD/UF/FO, fish fed 75PFAD then unfed for 7 days then fed FOFD for 21 days.

Table 5.6: Fatty acid composition (% of total FA) and lipid content (mg/g) of the viscera of Atlantic salmon fed either FO or 75PFAD during the grow-out period followed by either FOFD periods of 7 days and 28 days or 7 days food deprivation and 21 days FOFD periods

FA	Grow-out (day 77)						FOFD or unfed (day 84)						FOFD (day 105)					
	FO		75PFAD		FO		75PFAD/FO		75PFAD/UF		FO		75PFAD/FO		75%PFAD/UF/FO			
14:0	2.9	± 0.1 ^a	1.1	± 0.1 ^b	2.8	± 0.2 ^a	1.1	± 0.1 ^b	1.3	± 0.0 ^b	2.3	± 0.0	1.9	± 0.1	2.2	± 0.2		
16:0	16.1	± 0.1 ^b	17.6	± 0.3 ^a	16.6	± 0.1	16.7	± 0.4	17.7	± 0.4	16.3	± 0.1	17.4	± 0.2	17.5	± 0.4		
17:0	0.4	± 0.0 ^a	0.3	± 0.0 ^b	0.4	± 0.0 ^a	0.3	± 0.0 ^b	0.3	± 0.0 ^b	0.4	± 0.0 ^a	0.3	± 0.0 ^b	0.3	± 0.0 ^b		
18:0	4.0	± 0.0	4.4	± 0.1	4.1	± 0.0 ^b	4.5	± 0.1 ^a	4.3	± 0.0 ^{ab}	4.3	± 0.0	4.3	± 0.0	4.2	± 0.1		
16:1n-7c	8.9	± 0.0 ^a	4.7	± 0.1 ^b	9.3	± 0.0 ^a	5.2	± 0.1 ^b	4.8	± 0.1 ^b	8.9	± 0.0 ^a	6.0	± 0.2 ^b	6.4	± 0.1 ^b		
18:1n-7c	4.6	± 0.1 ^a	3.3	± 0.0 ^b	4.5	± 0.0 ^a	3.5	± 0.1 ^c	3.4	± 0.0 ^b	4.7	± 0.1 ^a	3.7	± 0.0 ^b	3.7	± 0.1 ^b		
18:1n-9c	14.2	± 0.2 ^b	33.8	± 0.4 ^a	14.9	± 0.1 ^c	30.9	± 0.6 ^b	33.8	± 0.2 ^a	15.4	± 0.2 ^b	28.4	± 1.0 ^a	28.1	± 0.6 ^a		
20:1n-7c	0.4	± 0.0 ^a	0.2	± 0.0 ^b	0.4	± 0.0 ^a	0.3	± 0.0 ^b	0.2	± 0.0 ^b	0.4	± 0.0 ^a	0.3	± 0.0 ^b	0.3	± 0.0 ^b		
20:1n-9	1.7	± 0.0 ^b	2.2	± 0.0 ^a	1.9	± 0.1 ^b	2.4	± 0.1 ^a	2.2	± 0.0 ^a	1.8	± 0.0 ^c	2.0	± 0.0 ^a	1.9	± 0.0 ^b		
22:1n-11c	0.8	± 0.1	0.6	± 0.1	0.9	± 0.0	0.8	± 0.1	0.7	± 0.0	0.8	± 0.0 ^a	0.6	± 0.0 ^b	0.6	± 0.0 ^b		
24:1n-9c	0.3	± 0.0	0.3	± 0.0	0.3	± 0.0	0.3	± 0.0	0.3	± 0.0	0.3	± 0.0	0.3	± 0.0	0.3	± 0.0		
18:2n-6	4.0	± 0.0 ^b	8.5	± 0.3 ^a	4.2	± 0.0 ^c	7.6	± 0.2 ^b	8.7	± 0.1 ^a	4.1	± 0.0 ^b	7.1	± 0.2 ^a	7.3	± 0.4 ^a		
20:4n-6	0.8	± 0.0 ^b	0.5	± 0.0 ^a	0.8	± 0.0 ^a	0.5	± 0.0 ^b	0.4	± 0.0 ^b	0.7	± 0.0 ^a	0.5	± 0.0 ^b	0.6	± 0.0 ^b		
18:3n-3	0.7	± 0.0	0.6	± 0.1	0.7	± 0.0 ^a	0.6	± 0.1 ^{ab}	0.4	± 0.0 ^b	0.7	± 0.0 ^a	0.7	± 0.0 ^a	0.6	± 0.1 ^b		
18:4n-3	2.7	± 0.0 ^a	1.3	± 0.0 ^b	2.7	± 0.0 ^a	1.6	± 0.1 ^b	1.3	± 0.0 ^c	2.7	± 0.1 ^a	1.6	± 0.2 ^b	1.7	± 0.1 ^b		
20:5n-3	11.7	± 0.3 ^a	4.4	± 0.1 ^b	10.9	± 0.1 ^a	5.4	± 0.3 ^b	4.5	± 0.1 ^b	11.3	± 0.1 ^a	6.4	± 0.2 ^b	6.6	± 0.3 ^b		
22:5n-3	4.2	± 0.1 ^a	2.4	± 0.1 ^b	4.2	± 0.0 ^a	2.8	± 0.1 ^b	2.5	± 0.1 ^c	4.3	± 0.0 ^a	2.9	± 0.1 ^b	2.9	± 0.1 ^b		
22:6n-3	11.9	± 0.1 ^a	7.8	± 0.0 ^b	11.5	± 0.1 ^a	9.0	± 0.1 ^b	7.7	± 0.1 ^c	11.9	± 0.0 ^a	8.7	± 0.2 ^b	8.3	± 0.2 ^b		
Total SFA	24.8	± 0.1	24.0	± 0.5	25.2	± 0.2 ^a	23.2	± 0.3 ^b	24.2	± 0.4 ^{ab}	24.5	± 0.2	24.9	± 0.2	25.0	± 0.3		
Total MUFA	32.0	± 0.3 ^b	45.8	± 0.1 ^a	33.3	± 0.2 ^c	44.1	± 0.4 ^b	46.0	± 0.1 ^a	33.2	± 0.1 ^b	42.1	± 0.7 ^a	41.7	± 0.3 ^a		
Total PUFA	43.2	± 0.4 ^a	30.2	± 0.5 ^b	41.5	± 0.1 ^a	32.7	± 0.7 ^b	29.9	± 0.3 ^c	42.3	± 0.3 ^a	33.0	± 0.6 ^b	33.3	± 0.4 ^b		
Total n-3	34.2	± 0.4 ^a	18.2	± 0.4 ^b	32.6	± 0.1 ^a	21.3	± 0.7 ^b	17.9	± 0.3 ^c	33.8	± 0.2 ^a	22.1	± 0.8 ^b	22.1	± 0.6 ^b		
Total n-6	6.1	± 0.1 ^b	10.7	± 0.3 ^a	5.9	± 0.1 ^c	9.8	± 0.1 ^b	10.6	± 0.1 ^a	5.8	± 0.0 ^b	9.2	± 0.3 ^b	9.4	± 0.4 ^b		
n-3 LC-PUFA	30.8	± 0.4 ^a	16.3	± 0.2 ^b	29.3	± 0.1 ^a	19.1	± 0.6 ^b	16.1	± 0.3 ^c	30.3	± 0.2 ^a	19.9	± 0.6 ^b	19.8	± 0.5 ^b		
n-3: n-6	5.6	± 0.1 ^a	1.7	± 0.1 ^b	5.5	± 0.1 ^a	2.2	± 0.1 ^b	1.7	± 0.0 ^c	5.9	± 0.0 ^a	2.4	± 0.2 ^b	2.4	± 0.2 ^b		
Lipid content (mg/g)																		
Dry	757.5	± 20.7	729.2	± 18.6	756.1	± 20.8	732.6	± 38.6	776.7	± 41.7	779.1	± 25.1	742.1	± 5.6	751.1	± 14.5		
Wet	380.6	± 29.1	369.8	± 12.8	399.6	± 24.2	370.2	± 20.7	391.4	± 10.7	404.2	± 4.4	393.3	± 9.2	411.7	± 10.5		

Values are means ± SEM, $n = 6$. Means in a row belonging to different feeding periods sharing different superscript letters were significantly different ($P < 0.05$). FO, fish fed FO; 75PFAD, fish fed 75PFAD; 75PFAD/FO, fish fed 75PFAD then fed FOFD; 75PFAD/UF, fish fed 75PFAD then unfed for 7 days; 75PFAD/UF/FO, fish fed 75PFAD then unfed for 7 days then fed FOFD for 21 days.

5.4.5 Regression analyses

The regression line between predicted (from dilution model) and observed %FA values for fillet and viscera were highly significant ($R^2 = 0.99$, $P < 0.001$) (Fig 5.3). The regression line had a strong degree of similarity with the line of equity, the line crossed at the origin and slope was close to 1. The regression equation is given as: Predicted = 0.1 + 0.98 Observed.

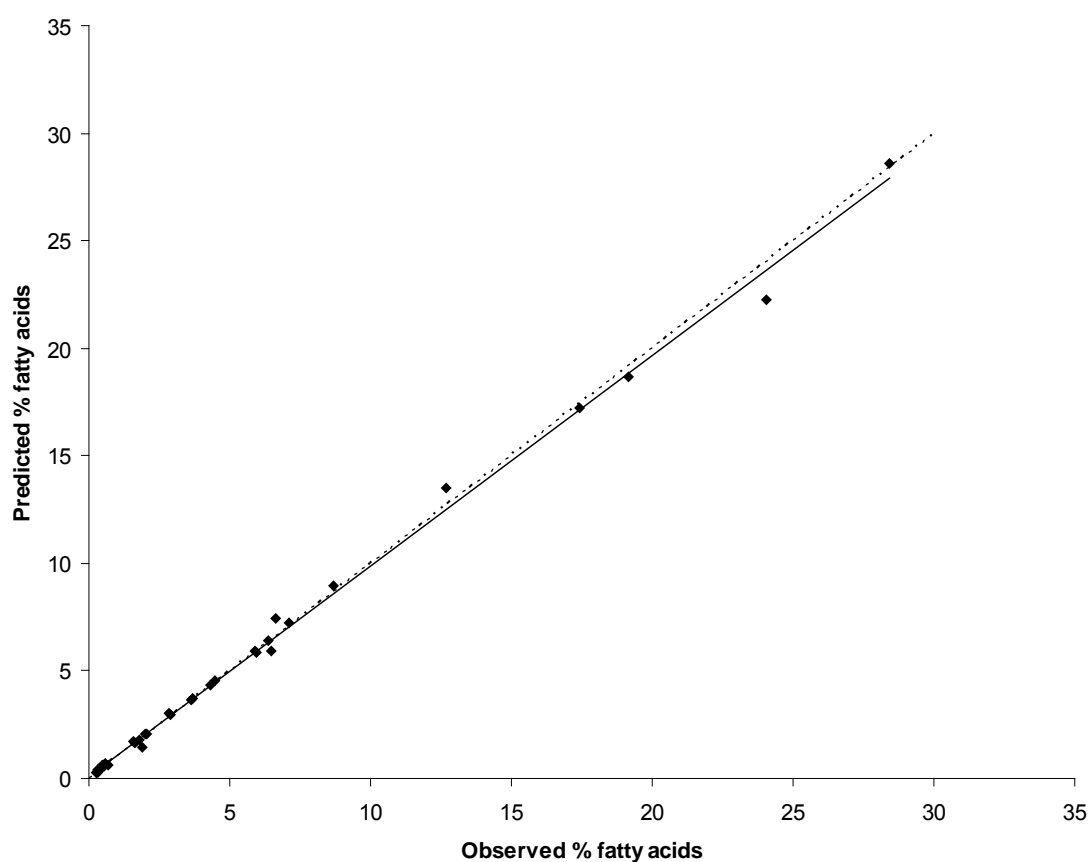


Figure 5.3: Total observed and predicted (from dilution model) fatty acid percentages (14:0, 16:0, 17:0, 18:0, 16:1n-7c, 18:1n-7c, 18:1n-9c, 20:1n-7c, 20:1n-9, 22:1n-11c, 24:1n-9c, 18:2n-6, 20:4n-6, 18:3n-3, 18:4n-3, 20:5n-3, 22:5n-3, 24:6n-3) in the fillet and viscera of Atlantic salmon after dietary shift from 75PFAD for 77 days to FO for 28 days (75PFAD/FO).

5.5 DISCUSSION

5.5.1 *Growth and FA apparent digestibility*

The evaluation of alternate oils to replace FO in aquafeeds for Atlantic salmon has been the subject of a range of studies (Miller et al., 2008; Turchini et al., 2009). Suitable candidate oils should be less expensive than FO and should not compromise fish health and growth. Various oils of vegetable origin have thus become popular substitutes for FO in aquafeeds. Palm-based oils are alternative oils characterised by high amounts of SFA. However, palm-based oils are not preferred FO substitutes in aquafeeds for cold water species such as Atlantic salmon due to the reduction in energy availability associated with the low digestibility of SFA (Ng et al., 2004). PFAD is rich in free fatty acids (~ 80%), and use of PFAD has been shown to markedly improve the AD of SFA in rainbow trout fed a diet where FO was substituted by 50% and 75% PFAD at 15°C and 20°C (Ng et al., 2010). In agreement with that study, it was shown that a 75PFAD improved the AD of SFA in Atlantic salmon, especially that of palmitic acid (16:0) which is the most abundant FA in PFAD. The high levels of free fatty acids in PFAD was identified as the reason for the high SFA digestibility by enhancing digestion and absorption of FA as opposed to SFA in the form of triacylglycerols (TAG) (Ng et al., 2010). In the present study, growth was not impaired by replacing 75% of FO with PFAD in the diet. The improvement in AD of SFA, in particular that of palmitic acid, in PFAD clearly increased energy availability. Surprisingly though, the AD of MUFA and PUFA by Atlantic salmon in our study was negatively affected by PFAD, which is in contrast to a FA digestibility study for rainbow trout (Ng et al., 2010). Although the AD of MUFA and PUFA was significantly lower, albeit by a small amount (~2%), the

relatively large improvement in AD of SFA (~ 11%) compensated for the small reduction in AD of MUFA and PUFA from the 75PFAD. It can be argued that since dietary SFA was high for 75PFAD and that the AD of SFA was increased hence increasing energy availability, that the growth of 75%PFAD fish should be higher than FO fish. However, FO diet had relatively high PUFA content with an AD of > 90% which compensates for the energy discrepancy *viz.* SFA digestible energy from 75 PFAD. A simple computation of total apparent digestible FA intake per fish during the grow-out period (average total food consumption x dietary FA composition x AD of individual FA) gives a total of 13.1g FA per fish for FO and 13.7g FA per fish for 75%PFAD. The AD of FA was highest for PUFA followed by MUFA and was lowest for SFA, which agrees with other FA digestibility studies for salmonids (Miller et al., 2007; Ng et al., 2010). Furthermore, AD generally decreased with increasing FA chain length and increased with increasing degree of unsaturation (Rosjo et al., 2000; Ng et al., 2004, 2010). These findings suggest that high dietary SFA from PFAD does not pose any restriction with Atlantic salmon smolt due to improved SFA digestibility. It is important to note that our experiment was performed at 15°C; this is typical of Tasmanian water temperatures and increasingly typical of temperatures experienced globally in Atlantic salmon aquaculture (Miller et al., 2006, 2008). It would be of interest to compare the digestibility and growth of Atlantic salmon fed a PFAD-based diet at lower temperatures.

5.5.2 FA profile – grow-out period

A common observation in all studies on FO substitution in aquafeeds is that the FA profile of fish generally mirrors the FA profile of the diet. In contrast, it was observed that the relative levels of SFA in Atlantic salmon fillet and viscera were not

different between FO and 75PFAD fish despite a 1.5 fold higher dietary SFA in 75PFAD. It has been suggested that suitable alternate oils should contain a certain proportion of SFA and MUFA (typically around 33% each) particularly palmitic acid and oleic acid (18:1n-9) because these FA are preferentially used for energy production by fish (Turchini et al., 2009). Since the net intake of SFA was higher for 75PFAD fish and SFA were not accumulated, SFA, particularly palmitic acid, were the preferred FA for β -oxidation. Palmitic acid was approximately 2 fold higher in the 75PFAD diet, but was only marginally higher in both fillet and viscera of 75PFAD fish.

5.5.3 FA profile – Fish oil finishing diet

Similar to all studies involving FO substitution in aquafeeds, the main drawback with use of PFAD remains the reduced levels of n-3 LC-PUFA in the fillet after a growth period with this VO-based diet. Feeding a FOFD for a certain period before harvest is a suitable way to restore n-3 LC-PUFA levels in fish (Pickova and Morkore, 2007). In the present study, 28 days feeding FOFD restored n-3 LC-PUFA levels in the fillet and viscera of 75PFAD fish to 72% and 66% of that of FO fish. The dynamics of n-3 LC-PUFA restoration in the fillet and viscera followed principally the dilution of existing FA stores and there was no evidence of preferential FA metabolism occurring. To achieve complete restoration of n-3 LC-PUFA, longer growth on the FOFD would be required which undermines the very purpose of FO substitution in aquafeeds (Turchini et al., 2009).

5.5.4 FA profile – short-term food deprivation followed by FOFD

Another strategy to improve the efficiency of n-3 LC-PUFA restoration when applying a FOFD is to reduce the initial lipid content in fish after growth on the alternate oil diet prior to commencing feeding on the FOFD (Palmeri et al., 2009). Food deprivation is one way to reduce the lipid content in fish. The way fish lose lipid seems to be species-specific and this has to be considered before applying this strategy (Palmeri et al., 2009). When deprived of food, Murray cod uses principally protein and hepatic lipid stores as their energy source (Palmeri et al., 2008, 2009). In a study with Murray cod, fish deprived of food for 5, 10 and 15 days did not lose lipid from the fillet and the hypothesis that lowering lipid content prior to feeding the FOFD would improve restoration of n-3 LC-PUFA could not be verified (Palmeri et al., 2009).

In Atlantic salmon, the fillet lipid content decreased most followed by that of the viscera and the liver after food deprivation (Einen et al., 1998). Consistent with these findings for Atlantic salmon in the present study there was a significant reduction in fillet lipid content (dry weight basis) during starvation. The reduced K value in unfed fish further supports this result. There was also a reduction in VSI of unfed fish which indicated a loss in visceral fat. However, there was no difference in visceral lipid content between fed and unfed fish. The probable reason for the “apparent” reduction in VSI in unfed fish was because fed fish might still contain residual feed in their gastrointestinal tract at sampling time compared to unfed fish. Due to the same reason, it was observed that in large Atlantic salmon (5 kg), the lipid content in the viscera of 7-86 days unfed fish was even higher compared to the fed group (Einen et al., 1998). The effect of lipid loss from the fillet of Atlantic salmon in

the present study was reflected by higher relative levels of n-3 LC-PUFA, specifically that of DHA in unfed fish compared to fed fish (albeit fish fed FO). Upon feed deprivation, fish used TAG rich lipid stores in the fillet for energy production, thus resulting in an increase in polar lipid (PL) relative to TAG. An increase in PL will lead to an increase in relative levels of n-3 LC-PUFA, particularly that of DHA which is abundant in PL. Subsequently, feeding a FOFD after a food deprivation period improved the efficiency of n-3 LC-PUFA restoration. However for absolute amounts of n-3 LC-PUFA, there was no significant difference between fish fed for 28 days with FOFD and fish unfed for 7 days then fed for 21 days with FOFD. During the FOFD period, fish were fed to satiation to compensate for growth and to restore total lipid content in unfed fish for 7 days. Feed consumption (g/fish) was lower for 75PFAD/UF/FO compared to 75PFAD/FO and FO fish (37.1 ± 10.0^b , 49.9 ± 2.1^a , 47.6 ± 0.1^a), though final weight, SGR and FER were not different. This was probably due to large variations in fish size especially for the re-feeding of unfed fish after 7 days. As opposed to the fillet, the FA composition in unfed fish was similar to the composition in the viscera of fish fed 75% PFAD after grow-out (75% PFAD). In harvest size Atlantic salmon after food deprivation periods ranging from 7 to 86 days, the lipid level in the viscera was relatively constant (Einen et al., 1998). In the present study, there were higher relative levels of n-3 LC-PUFA inclusive of DHA in fish fed for 7 days FOFD (PFAD/FO) compared to 7 days without feeding (PFAD/UF). This suggests that depletion of FA stores in early stages of food deprivation did not occur in the viscera and the deposition of new FA stores occurred in the viscera immediately after the switch to a new diet. When fed to satiation on the FOFD for 21 days after 7 days food deprivation, the relative levels of n-3 LC-PUFA were similar to that of the fish fed for 28 days.

As highlighted earlier, a key element in the restoration of n-3 LC-PUFA in Atlantic salmon is the reduction of initial lipid content in the fillet which can be achieved by a food deprivation period. This present study is conceptual and may not be reflective of or be applicable to harvest size Atlantic salmon. Since the energy requirement in unfed fish depends on water temperature and body weight (Cho and Bureau, 1995), the loss of lipid in the fillet of fish will increase with temperature and decrease with body weight. In this study, Atlantic salmon were ~ 150 g and the temperature was 15°C. Both the fish weight and temperature were favourable for significant lipid loss in the fillet after 7 days of food deprivation. In larger Atlantic salmon of ~5 kg weight and with temperature averaging 4.1°C, there was a significant reduction (1.4%) in lipid content of the muscle after 58 and 86 days of food deprivation. In Atlantic salmon of 3.5 kg weight at temperatures ranging from 3.2 to 4.9°C, there was a reduction of fillet fat content (2-3%) after 110 days of food deprivation (Einen et al., 1999). In Atlantic salmon of 2 kg weight, there was a more pronounced reduction in fillet fat (2-4%) after 35 and 78 days of food deprivation (Lie and Huse, 1992). Since the harvest size of Atlantic salmon is generally around 4 kg and feeding FOFD should occur a few months prior to harvest, it is very likely that several months of food deprivation would be needed to significantly reduce the lipid content in fish fillet. However, as temperature will affect the reduction in lipid content, a higher temperature, such as that in this present study (15°C) and as now commonly occurs in Tasmanian waters in the summer period, may shorten the food deprivation period. In light of the above observations, the next steps to attempt to improve the efficiency of n-3 LC-PUFA restoration in harvest size Atlantic salmon by using a FOFD should include targeting summer months for a food deprivation period.

5.6 CONCLUSION

A 75% FO substitution by PFAD in the diet of Atlantic salmon smolt did not impair fish growth and the AD of SFA was markedly improved using 75PFAD. Feeding with 75PFAD for 77 days resulted in lower relative levels and absolute amounts of n-3 LC-PUFA in the fillet. Subsequent feeding on a FOFD for 28 days restored n-3 LC-PUFA relative levels and absolute amounts to 72% and 71% respectively, of that of fish fed FO throughout. It was also shown that a short term food deprivation period of 7 days prior to feeding a FOFD for 21 days improved n-3 LC-PUFA restoration (to 81% and 80% for relative levels and absolute amounts, respectively) in the fillet of fish previously fed on 75 PFAD.

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CHAPTER 6

Effect of higher dietary DHA to EPA ratio and lower dietary n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid (n-3 LC-PUFA) content to that common in typical fish oil diet on n-3 LC-PUFA deposition in muscle and liver of Atlantic salmon smolt.

Adapted from Codabaccus, M.B., Bridle, A.R., Nichols, P.D., Carter, C.G., 2011. Effect of higher dietary DHA to EPA ratio and lower dietary n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid (n-3 LC-PUFA) content to that common in typical fish oil diets on n-3 LC-PUFA deposition in muscle and liver of Atlantic salmon smolt. *In preparation*.

6.1 ABSTRACT

Fish oil (FO) is a major source of the human health benefitting n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA), particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) via consumption of farmed fish. Use of alternate oils (AO) for FO substitution decreases the content of n-3 LC-PUFA in aquafeeds, with blends of AO and FO in aquafeeds for Atlantic salmon is a common practice. The n-3 LC-PUFA, in particular EPA, is used extensively by fish for β -oxidation when in dietary surplus. Therefore it is of interest to optimize the deposition of n-3 LC-PUFA in fish by aiming at “saving” n-3 LC-PUFA through reduction of n-3 LC-PUFA β -oxidation. In this context, both the absolute and relative amounts of dietary EPA and DHA are important. This study tested whether Atlantic salmon smolt fed higher a dietary DHA: EPA ratio as well as lower dietary n-3 LC-PUFA content to that of a FO diet would optimize deposition of n-3 LC-PUFA in the liver and muscle. Comparisons were made between fish fed a FO diet, a blend of 50% rapeseed and 50% tuna oils diet (model oil, MO 1), a blend of 50% rapeseed, 25% tuna and 25% FO diet (MO 2), a blend of 50% FO and 50% chicken fat diet (FO/CF). The dietary DHA: EPA ratio was in the order MO 1 > MO 2 > FO/CF ~ FO and dietary n-3 LC-PUFA content was approximately 2-fold lower in MO1, MO2 and FO/CF compared to the FO diet. Comparable amounts of n-3 LC-PUFA to FO fish was obtained in the muscle for MO 1 (highest dietary DHA: EPA ratio and lowest dietary n-3 LC-PUFA content) and FO/CF fish. In the liver there was no difference in n-3 LC-PUFA content between all treatments. This study indicates that both the absolute and relative amounts of EPA and DHA required to optimize deposition of n-3 LC-PUFA through “saving” n-3 LC-PUFA that are otherwise prone to β -oxidation.

6.2 INTRODUCTION

Fish oil (FO) substitution in aquafeeds for Atlantic salmon has become unavoidable due to the limited global supply of FO (Naylor et al., 2009). The use of alternate oils (AO) from plants and/or as rendered animal fat as a substitute for FO in aquafeeds results in altered fatty acid (FA) composition of farmed Atlantic salmon, especially that of the health-benefitting n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA). Since AO lack n-3 LC-PUFA, the content of n-3 LC-PUFA in fish generally decreases with increasing levels of FO substitution in aquafeeds. Though FO may be completely substituted by AO in diets for Atlantic salmon (Bell et al., 2005, Torstensen et al., 2005), such practice is not recommended so as to not compromise the human health benefits of consuming fish rich in n-3 LC-PUFA (Sieirstad, 2005). Enhanced understanding of the lipid metabolism of Atlantic salmon has enabled significant advancement with the choice of AO in blends for inclusion in diets. In Atlantic salmon, eicosapentaenoic acid (EPA; 20:5n-3) is extensively used for β -oxidation when supplied in dietary surplus (Stubhaug et al., 2007; Chapter 2 and Codabaccus et al., 2011) and docosahexaenoic acid (DHA; 22:6n-3) is mostly conserved irrespective of its dietary concentration (Tocher, 2010). Saturated FA (SFA) and monounsaturated FA (MUFA) are preferred substrates for β -oxidation (Turchini et al., 2009) although their inclusion in diets may be of concern for cold water fish species because the AD is lower than polyunsaturated FA (PUFA) (Ng et al., 2004). In addition, endogenous biosynthesis of n-3 LC-PUFA from its biosynthetic precursor α -linolenic acid (ALA; 18:3n-3) is inefficient in the marine environment (Sargent et al., 2002). All the above suggest that a suitable diet for Atlantic salmon should comprise a certain proportion of SFA and/or MUFA for energy production, EPA should not be present at a dietary surplus, whereas DHA can

be maintained at a relatively high level. The proportion of SFA and/or MUFA in diets, which is typically around 33% for each of these two FA classes (Turchini et al., 2009), can be easily achieved with available AO, with the remaining consisting of PUFA. For the PUFA, especially the n-3 LC-PUFA, this dietary component cannot be met by AO, as the only commercially viable source of the biologically important n-3 LC-PUFA, EPA and DHA, available for aquafeeds is from FO obtained from wild caught fish. The proportion of these different FA classes in aquafeeds is generally based using a FO diet as a benchmark. However, such proportions might not be the most suitable for efficient deposition of n-3 LC-PUFA in fish, as a result of an unbalanced DHA: EPA ratio in oil blends and surplus dietary EPA present being readily β -oxidised. The ratio of DHA: EPA is generally 1:1.5 with variations occurring depending on the geographical distribution or seasonal differences in the catch of wild fish, especially those from the southern hemisphere having higher EPA (Pratoomyot et al., 2008). As a consequence, blending FO with any other AO essentially provides a ratio around 1: 1.5 for DHA: EPA, with EPA in surplus. Based on the above criteria, an improved scenario would be to have a higher ratio of DHA: EPA. In the field of plant genomics, recent advances have shown that EPA and DHA can be produced by genetically engineering oil seed crops and such oils may become a potential alternate to FO in aquafeeds in the near future (Nichols et al., 2010; Petrie et al. 2010a; Venegas-Caleron et al., 2010). An interesting characteristic of oils from a genetically modified model crop according to current research is that the percentage of DHA may be higher than EPA (Petrie et al., 2010b). As highlighted above, this feature may be favourable when formulating diets and the effect of such diets on n-3 LC-PUFA deposition in Atlantic salmon will be of interest. Since oils from genetically modified plants having a higher DHA: EPA ratio are not presently

available, two oils were blended, rapeseed oil and tuna oil to obtain model oils (MO) containing markedly higher DHA to EPA ratios than is commonly used. The objective of the experiment was to investigate the deposition of n-3 LC-PUFA in the muscle and liver of Atlantic salmon fed two MO containing diets with the levels of DHA and EPA used being those likely to be achieved in the near future in land plants and both exhibiting a DHA:EPA ratio of > 1. Comparisons were made to a FO-based diet and a diet comprising a commercially practised oil blend of FO and chicken fat (CF) as used in aquafeeds for Atlantic salmon grown in some regions such as Tasmania (Australia).

6.3 MATERIALS and METHODS

6.3.1 Experimental diets

Four isonitrogenous and isolipidic diets were formulated in which the added lipid source varied; a 100% fish oil, Jack mackerel, *Trachurus symmetricus* L. (FO), a blend of 50% rapeseed and 50% tuna oils (MO 1), a blend of 50% rapeseed, 25% tuna oil and 25% Jack mackerel, *Trachurus symmetricus* L. FO (MO 2), a blend of 50% Jack mackerel, *Trachurus symmetricus* L. FO and 50% chicken fat (FO/CF) (Table 6.1). Diets were manufactured into 3 mm diameter pellets using a California Pellet Mill (CL-2, San Francisco, CA, USA), dried and stored at -5°C until use (Carter et al. 2003).

6.3.2 Growth experiment

The experiment was conducted at the University of Tasmania (Launceston, Tasmania, Australia) in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0009731). Atlantic salmon (*Salmo salar* L.) smolts of average weight ~ 70 g (Wayatinah Salmon Hatchery, SALTAS, Tasmania, Australia),

were acclimated in seawater for a period of 14 days before the experiment. Fish were fed a commercial diet (Skretting, Tasmania, Australia) during the acclimation period. After acclimation, fish were randomly distributed in twelve 300 L fibreglass tanks at a stocking density of 35 fish per tank. Rearing tanks were connected to a partial recirculating system equipped with a protein skimmer and physical, UV and biological filters (Carter and Hauler, 2000). At the start of the experiment, fish were anaesthetized (50 mg/L, benzocaine) and bulk weighed. Six fish were euthanized (100 mg/L), the dorsal muscle and liver were dissected and samples stored at -20°C for measurement of initial lipid content and FA composition. Fish were fed one of the four experimental diets (3 replicate tanks/diet) at a fixed ration of 1.5% body weight/d (BW/d) in two equal daily rations for a period of 75 days. Water temperature was kept constant at 15 °C. Every 14 days fish were bulk weighed to readjust the feed ration.

At the end of 75 days growth on the different diets, fish were bulk weighed. Muscle and liver from three fish per tank (12 fish/treatment) were dissected and stored at -20°C prior to analysis of lipid content and FA composition. All samples were pooled on a per tank basis for chemical analysis ($n = 3$).

6.3.3 Chemical analysis

Standard methods were used to determine dry matter (DM) (freeze dry to constant weight then drying at 135°C for 2 h) (AOAC, 1995) of experimental diets; total lipid (Bligh & Dyer, 1959); nitrogen (Kjeldahl using selenium catalyst; crude protein was calculated as $N \times 6.25$); energy (bomb calorimeter, Gallenkamp

Autobomb, calibrated with benzoic acid) and ash by combustion at 600°C for 2 h (AOAC, 1995). Apart from DM, freeze dried samples were used for all analyses.

Table 6.1: Ingredient and chemical composition (g/kg DM) of experimental diets

	Diet			
	FO	MO 1	MO 2	FO/CF
<i>Ingredient composition (g/ kg)</i>				
Fishmeal ¹	300	300	300	300
Casein ²	50	50	50	50
Wheat gluten ³	100	100	100	100
Soybean meal ⁴	139	139	139	139
Jack mackerel oil ¹	200	0	50	100
Tuna oil ⁵	0	100	100	0
Rapeseed oil ⁶	0	100	50	0
Chicken fat ¹	0	0	0	100
Pre-gel starch ⁷	127	127	127	127
Vitamin mix ⁸	7	7	7	7
Mineral mix ⁹	7	7	7	7
Stay-C ¹⁰	6	6	6	6
Choline chloride ¹¹	2	2	2	2
Sipernat ¹²	40	40	40	40
CMC ¹¹	10	10	10	10
Monobasic calcium phosphate ¹¹	20	20	20	20
Yttrium oxide ¹¹	1	1	1	1
<i>Chemical composition</i>				
Dry matter (g/kg)	916.6	919.9	912.2	915.6
Crude protein	353.2	355.1	350.0	358.7
Total lipid	235.1	233.6	238.2	237.4
Ash	105.2	104.3	107.6	102.8
Energy (MJ/kg)	19.8	19.8	19.9	19.9

FO, 100% fish oil (Jack mackerel); MO 1, a blend of 50% rapeseed and 50% tuna oils; MO 2, a blend of 50% rapeseed, 25% tuna and 25% FO (Jack mackerel); FO/CF, a blend of 50% FO (Jack mackerel) and 50% chicken fat. ¹Skretting Australia, Cambridge, Tasmania, Australia; ²MP Biomedicals Australasia Pty. Ltd., Seven Hills NSW, Australia; ³Starch Australasia, Lane Cove, NSW, Australia; ⁴Hamlet Protein A/S, Horstens, Denmark; ⁵Wilmar Edible Oils Ltd., Penang, Malaysia; ⁷Penford Limited, Lane Cove, NSW, Australia; ⁸Vitamin mix (ASV4) as listed in Carter et al. (2003); ⁹Mineral mix (TMV4) as listed in Carter et al. (2003); ¹⁰L-Ascorbyl-2-polyphosphate (Roche Vitamins Australia, Frenchs Forest, NSW, Australia); ¹¹Sigma-Aldrich, Castle Hill, NSW, Australia, ¹²Degussa, Frankfurt, Germany.

6.3.4 Lipid extraction and fatty acid analyses

Diets, muscle and liver samples were freeze dried and extracted overnight using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a

single phase extraction using CHCl₃/MeOH/H₂O (1:2:0.8, v/v/v), followed by phase separation to yield a total lipid extract (TLE).

An aliquot of the TLE was trans-methylated in methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 2 h at 100°C. After addition of MilliQ water (1 ml), the mixture was extracted with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). Samples with an internal injection standard (19:0 FAME) added were analysed by gas chromatography (GC) using an Agilent Technologies 7890B GC (Palo Alto, California, USA) equipped with a non-polar Equity™-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 µm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683 B Series auto sampler. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 120°C. After injection, oven temperature was raised to 270°C at 10°C/min and finally to 310°C at 5°C/min. Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, California, USA). GC results are typically subject to an error of up to ± 5% of individual component area.

Individual components were identified by mass spectral data and by comparing retention time data with authentic and laboratory standards. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, Texas, USA). The GC was equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.) of similar polarity to that described above. Helium was used as carrier gas, with operating conditions previously described (Miller et al., 2006).

6.3.5 Statistical analysis

Specific growth rate (SGR) was calculated as $SGR (\%/d) = 100 * (\ln W_f / \ln W_i) / d$ where W_f and W_i are the final and initial weights (g) and d is the number of days of the experiment. Feed consumption (FC) was calculated as the average total amount of dry feed (g DM) consumed per tank over the experiment. The feed efficiency ratio was calculated as $FER (g/g) = \text{total weight gain (g)} / FC (g DM)$. Values are reported as mean \pm standard error of the mean (SEM). Normality and homogeneity of variance were confirmed prior to analysis. Pooled samples from individual tanks ($n = 3$) were compared between treatment means for lipid content, FA content and growth performance by one way ANOVA followed by multiple comparisons using Tukey-Kramer HSD wherever applicable. Significance was accepted at probabilities $P < 0.05$. Statistical analysis was performed using SPSS for Windows version 16.0.

6.4 RESULTS

6.4.1 Lipid analyses – diet

In both model oil - MO 1 and MO 2 - diets, there was higher DHA than EPA content with DHA: EPA ratios of 2.8 and 1.3 respectively. In the FO and FO/CF diets, the DHA content was lower than that of EPA with DHA: EPA ratio of 0.5 (Table 6.2). MO1 had the lowest n-3 LC-PUFA content and FO had the highest n-3 LC-PUFA content. The dominant FA class in the MO 1 and MO 2 diets was MUFA, particularly oleic acid (OA; 18:1n-9c). In the FO/CF diet, MUFA was also dominant especially OA. In FO diet, PUFA was the dominant FA class, particularly the n-3 series. The dominant FA in the FO diet was palmitic acid (16:0).

Table 6.2: Fatty acid content (mg/g DM) of experimental diets

FA	FO		MO1		MO2		FO/CF	
14:0	6.1	± 0.4 ^a	2.0	± 0.1 ^c	2.5	± 0.1 ^{bc}	3.9	± 0.6 ^b
16:0	37.4	± 0.8 ^a	22.0	± 0.1 ^b	24.4	± 0.3 ^b	36.1	± 0.8 ^a
17:0	0.9	± 0.0 ^b	1.1	± 0.0 ^a	0.9	± 0.0 ^b	0.6	± 0.0 ^c
18:0	7.8	± 0.2 ^b	7.7	± 0.0 ^b	7.3	± 0.1 ^b	9.4	± 0.1 ^a
Other SFA ¹	1.7	± 0.2 ^b	2.3	± 0.1 ^a	2.1	± 0.0 ^{ab}	0.9	± 0.0 ^c
16:1n-7c	17.3	± 0.5 ^a	4.2	± 0.0 ^d	7.1	± 0.1 ^c	13.5	± 0.4 ^b
18:1n-7c	7.5	± 0.2 ^a	7.2	± 0.0 ^a	7.4	± 0.1 ^a	6.7	± 0.1 ^b
18:1n-9c	20.6	± 0.4 ^d	75.1	± 0.2 ^a	65.1	± 0.8 ^b	52.4	± 0.5 ^c
20:1n-7c	0.7	± 0.1 ^a	0.1	± 0.1 ^b	0.3	± 0.0 ^b	0.3	± 0.0 ^b
20:1n-9	1.8	± 0.1 ^c	2.8	± 0.1 ^a	2.5	± 0.0 ^b	1.5	± 0.0 ^c
22:1n-11c	1.1	± 0.0 ^a	0.4	± 0.0 ^b	0.7	± 0.1 ^b	0.7	± 0.1 ^b
24:1n-9c	0.7	± 0.0 ^a	0.7	± 0.0 ^a	0.7	± 0.0 ^a	0.4	± 0.0 ^b
Other MUFA ²	2.1	± 0.3	2.0	± 0.0	1.6	± 0.0	2.0	± 0.0
18:2n-6	6.8	± 0.2 ^d	26.7	± 0.1 ^a	23.1	± 0.3 ^b	16.9	± 0.2 ^c
20:4n-6	1.6	± 0.0 ^a	1.4	± 0.0 ^b	1.3	± 0.1 ^b	1.1	± 0.0 ^c
Other n-6	1.2	± 0.1 ^b	1.7	± 0.0 ^a	1.4	± 0.1 ^{ab}	0.8	± 0.0 ^c
18:3n-3	1.3	± 0.0 ^d	9.3	± 0.0 ^a	8.0	± 0.1 ^b	2.8	± 0.0 ^c
18:4n-3	4.9	± 0.1 ^a	0.7	± 0.0 ^d	1.8	± 0.0 ^c	2.8	± 0.1 ^b
20:4n-3	1.7	± 0.0 ^a	0.6	± 0.0 ^c	0.7	± 0.0 ^{bc}	0.9	± 0.0 ^b
20:5n-3; EPA	33.7	± 0.7 ^a	6.9	± 0.0 ^d	12.6	± 0.1 ^c	18.4	± 0.1 ^b
22:5n-3	3.9	± 0.1 ^a	1.3	± 0.0 ^d	1.8	± 0.0 ^c	2.2	± 0.1 ^b
22:6n-3; DHA	16.9	± 0.4 ^b	19.3	± 0.2 ^a	16.1	± 0.3 ^b	10.1	± 0.0 ^c
Other n-3	1.4	± 0.0 ^a	0.2	± 0.0 ^d	0.5	± 0.1 ^c	0.7	± 0.0 ^b
Other PUFA ³	7.8	± 0.2 ^a	0.2	± 0.0 ^d	2.4	± 0.1 ^c	4.1	± 0.2 ^b
Total SFA	53.9	± 1.5 ^a	35.0	± 0.2 ^b	37.3	± 0.3 ^b	50.9	± 1.5 ^b
Total MUFA	51.7	± 1.5 ^d	92.7	± 0.3 ^a	85.3	± 1.0 ^b	77.7	± 1.0 ^c
Total PUFA	81.1	± 2.0 ^a	68.4	± 0.3 ^b	69.7	± 1.0 ^b	60.8	± 0.7 ^c
Total n-3	63.8	± 1.5 ^a	38.4	± 0.3 ^b	41.5	± 0.6 ^b	37.9	± 0.3 ^b
Total n-6	9.5	± 0.3 ^d	29.7	± 0.0 ^a	25.8	± 0.5 ^b	18.7	± 0.2 ^c
n-3 LC-PUFA	57.6	± 1.3 ^a	28.4	± 0.3 ^c	31.7	± 0.5 ^b	32.3	± 0.1 ^b
DHA: EPA	0.5	± 0.0 ^c	2.8	± 0.0 ^a	1.3	± 0.0 ^b	0.5	± 0.0 ^c
n-3:n-6	6.7	± 0.1 ^a	1.3	± 0.0 ^d	1.6	± 0.0 ^c	2.0	± 0.0 ^b

FO, 100% fish oil (Jack mackerel); MO 1, a blend of 50% rapeseed and 50% tuna oils; MO 2, a blend of 50% rapeseed, 25% tuna and 25% FO (Jack mackerel); FO/CF, a blend of 50% FO (Jack mackerel) and 50% chicken fat. Values are means ± SEM, $n = 3$. Means in a row sharing different superscript letters were significantly different ($P < 0.05$). ¹Saturated fatty acids, ²Monounsaturated fatty acids, ³Polyunsaturated fatty acids.

6.4.2 Growth

Dietary oil source had no significant ($P > 0.05$) effect on final weight, SGR and FER at the end of 75 days growth (Table 6.3).

Table 6.3: Growth parameters of Atlantic salmon smolt grown on different dietary lipid sources for 75 days

Diet	Initial weight (g)	Final weight (g)	SGR (% BW)	FER (g/g)
FO	71.5 ± 1.9	147.9 ± 4.1	1.0 ± 0.0	1.0 ± 0.0
MO 1	70.8 ± 1.0	153.4 ± 5.6	1.0 ± 0.0	1.1 ± 0.0
MO 2	72.2 ± 0.6	155.3 ± 2.1	1.0 ± 0.0	1.0 ± 0.0
FO/CF	73.5 ± 1.1	156.9 ± 2.2	1.0 ± 0.0	1.0 ± 0.0

FO, 100% fish oil (Jack mackerel); MO 1, a blend of 50% rapeseed and 50% tuna oils; MO 2, a blend of 50% rapeseed, 25% tuna and 25% FO (Jack mackerel); FO/CF, a blend of 50% FO (Jack mackerel) and 50% chicken fat. Values are means ± SEM, $n = 3$.

6.4.3 Lipid analyses – white muscle

There was no significant ($P > 0.05$) difference in total SFA and total PUFA content between dietary treatments (Table 6.4). MO 1 fish had the highest MUFA content and FO fish had the lowest MUFA content. Total n-3 content was not significantly different between dietary treatments. Total n-6 content was highest for MO 1 fish and lowest for FO fish. FO fish had significantly ($P < 0.05$) higher EPA content than the other treatments. DHA content was not different between dietary treatments. The n-3 LC-PUFA content was not different between FO, MO 1 and FO/CF fish, with the MO 2 fish having lower n-3 LC-PUFA content. The DHA: EPA ratio was 1.4, 3.6, 2.8, and 1.9 for FO fish, MO 1 fish, MO 2 fish and FO/CF fish respectively. FO fish had the highest n-3: n-6 ratio.

6.4.4 Lipid analyses – liver

There was no significant ($P > 0.05$) difference in total SFA, total MUFA and total PUFA between dietary treatments (Table 6.5). Total n-3 content was not different between dietary treatments. Total n-6 content was highest for MO 1 fish and lowest for FO fish. EPA content was not significantly ($P > 0.05$) different between FO and FO/CF fish and the EPA content of those two treatments was higher than that of MO 1 and MO 2 fish. DHA content was not different between dietary treatments. Similarly, n-3 LC-PUFA was not different between dietary treatments. The DHA:EPA ratio was 2.1, 6.6, 4.0, and 3.0 for FO fish, MO 1 fish, MO 2 fish and FO/CF fish respectively and FO fish had the highest n-3: n-6 ratio.

6.4.5 Changes in dietary and tissue EPA and DHA concentrations

There was significantly ($P < 0.05$) smaller difference (on a % basis) – between dietary and muscle EPA concentrations for MO 1 treatment compared to the other treatments (Fig 6.1). There was significantly ($P < 0.05$) smaller difference between dietary and liver EPA concentrations for both MO 1 and MO 2 treatments compared to FO treatment. There was significantly ($P < 0.05$) smaller difference between dietary and muscle DHA concentrations for FO/CF treatment compared to the other treatments. The difference between dietary and liver DHA was highest for the MO 1 treatment compared to the MO 2 and FO treatments, with negative differences obtained. A positive difference between dietary and liver DHA was obtained for the FO/CF treatment.

Table 6.4: Fatty acid content (mg/g DM) of the muscle of Atlantic salmon smolt fed on different dietary oils for 75 days

FA	Initial	FO	MO1	MO2	FO/CF
14:0	2.5 ± 0.0	1.6 ± 0.2	0.9 ± 0.1	1.1 ± 0.2	1.4 ± 0.2
16:0	16.6 ± 0.5	13.7 ± 1.3	12.2 ± 1.3	10.9 ± 0.2	14.4 ± 2.0
17:0	0.3 ± 0.0	0.3 ± 0.0 ^b	0.5 ± 0.0 ^a	0.3 ± 0.0 ^b	0.3 ± 0.0 ^b
18:0	4.5 ± 0.1	3.6 ± 0.2	4.3 ± 0.4	3.4 ± 0.1	4.3 ± 0.5
Other SFA	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.2
16:1n-7c	6.8 ± 0.9	5.9 ± 0.7 ^a	3.1 ± 0.4 ^b	3.0 ± 0.1 ^b	5.2 ± 0.8 ^{ab}
18:1n-7c	4.4 ± 0.1	3.4 ± 0.3	3.6 ± 0.4	2.9 ± 0.0	3.3 ± 0.5
18:1n-9c	15.8 ± 0.3	10.5 ± 1.1 ^b	28.5 ± 3.5 ^a	20.6 ± 0.5 ^{ab}	20.2 ± 3.0 ^{ab}
20:1n-7c	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
20:1n-9	2.6 ± 0.1	1.2 ± 0.2	2.0 ± 0.3	1.4 ± 0.0	1.5 ± 0.2
22:1n-11c	1.3 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.5 ± 0.1
24:1n-9c	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
Other MUFA	0.9 ± 0.1	0.6 ± 0.0	1.1 ± 0.1	0.7 ± 0.1	0.7 ± 0.2
18:2n-6	3.7 ± 0.2	2.8 ± 0.3 ^c	8.9 ± 1.0 ^a	6.6 ± 0.2 ^{ab}	5.6 ± 0.8 ^{bc}
20:4n-6	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.1
Other n-6	0.6 ± 0.0	0.7 ± 0.1 ^b	1.4 ± 0.2 ^a	1.0 ± 0.0 ^{ab}	0.8 ± 0.1 ^b
18:3n-3	0.6 ± 0.0	0.5 ± 0.0 ^b	2.6 ± 0.3 ^a	1.9 ± 0.1 ^a	0.8 ± 0.1 ^b
18:4n-3	2.3 ± 0.0	1.8 ± 0.2 ^a	0.8 ± 0.1 ^b	0.9 ± 0.0 ^b	1.3 ± 0.2 ^{ab}
20:4n-3	0.8 ± 0.1	0.8 ± 0.1 ^a	0.5 ± 0.0 ^b	0.4 ± 0.0 ^b	0.6 ± 0.1 ^{ab}
20:5n-3; EPA	8.0 ± 0.2	8.6 ± 0.6 ^a	3.5 ± 0.4 ^b	3.9 ± 0.1 ^b	5.3 ± 0.7 ^b
22:5n-3	3.0 ± 0.5	3.2 ± 0.2 ^a	1.5 ± 0.2 ^b	1.5 ± 0.0 ^b	2.2 ± 0.3 ^b
22:6n-3; DHA	12.8 ± 0.8	12.2 ± 0.7	12.5 ± 1.0	10.8 ± 0.4	10.0 ± 1.1
Other n-3	1.9 ± 0.0	1.1 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
Other PUFA	2.6 ± 0.1	1.8 ± 0.1 ^a	0.6 ± 0.2 ^b	0.8 ± 0.0 ^b	1.2 ± 0.2 ^{ab}
Total SFA	24.7 ± 0.5	20.0 ± 1.7	18.5 ± 2.0	16.3 ± 0.2	20.9 ± 2.9
Total MUFA	32.5 ± 1.5	22.7 ± 2.4 ^c	39.4 ± 4.6 ^a	29.5 ± 0.6 ^{ab}	31.9 ± 4.7 ^{ab}
Total PUFA	36.8 ± 1.8	34.1 ± 2.3	33.8 ± 3.3	29.0 ± 0.2	29.2 ± 3.8
Total n-3	29.1 ± 1.7	28.2 ± 1.9	22.2 ± 2.1	20.2 ± 0.4	21.1 ± 2.6
Total n-6	4.9 ± 0.3	4.1 ± 0.3 ^c	11.0 ± 1.2 ^a	8.1 ± 0.2 ^{ab}	6.9 ± 1.0 ^{bc}
n-3 LC-PUFA	26.3 ± 1.6	25.9 ± 1.6 ^a	18.8 ± 1.8 ^{ab}	17.3 ± 0.5 ^b	19.0 ± 2.3 ^{ab}
DHA: EPA	1.6 ± 0.0	1.4 ± 0.0 ^d	3.6 ± 0.1 ^a	2.8 ± 0.0 ^b	1.9 ± 0.1 ^c
n-3:n-6	5.9 ± 0.0	6.8 ± 0.1 ^a	2.0 ± 0.1 ^c	2.5 ± 0.1 ^c	3.1 ± 0.1 ^b

Values are means ± SEM, $n = 3$. Means in a row sharing different superscript letters were significantly different ($P < 0.05$). FO, 100% fish oil (Jack mackerel); MO 1, a blend of 50% rapeseed and 50% tuna oils; MO 2, a blend of 50% rapeseed, 25% tuna and 25% FO (Jack mackerel); FO/CF, a blend of 50% FO (Jack mackerel) and 50% chicken fat.

Table 6.5: Fatty acid content (mg/g DM) of the liver of Atlantic salmon smolt fed on different dietary oils for 75 days

FA	Initial	FO	MO1	MO2	FO/CF
14:0	2.0 ± 0.6	0.7 ± 0.2	0.2 ± 0.1	0.5 ± 0.2	0.4 ± 0.1
16:0	22.9 ± 2.3	9.2 ± 1.5	7.6 ± 0.7	8.2 ± 1.3	8.3 ± 1.2
17:0	0.4 ± 0.1	0.2 ± 0.0 ^b	0.3 ± 0.0 ^a	0.3 ± 0.0 ^a	0.2 ± 0.0 ^b
18:0	6.3 ± 0.6	4.0 ± 0.2	3.5 ± 0.2	3.5 ± 0.2	3.5 ± 0.4
Other SFA	0.7 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0
16:1n-7c	6.6 ± 1.6	2.2 ± 0.7	0.8 ± 0.1	1.1 ± 0.2	1.6 ± 0.4
18:1n-7c	4.9 ± 0.8	2.2 ± 0.3	1.5 ± 0.1	1.8 ± 0.2	1.8 ± 0.2
18:1n-9c	16.7 ± 3.0	6.4 ± 1.0 ^b	11.2 ± 0.4 ^a	11.1 ± 0.9 ^a	9.4 ± 1.0 ^{ab}
20:1n-7c	0.2 ± 0.1	0.2 ± 0.0 ^a	0.1 ± 0.0 ^b	0.1 ± 0.0 ^b	0.1 ± 0.0 ^b
20:1n-9	2.2 ± 0.6	0.8 ± 0.1 ^b	1.4 ± 0.1 ^a	1.2 ± 0.1 ^{ab}	1.1 ± 0.0 ^{ab}
22:1n-11c	1.1 ± 0.3	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
24:1n-9c	1.5 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.0 ± 0.1
Other MUFA	1.3 ± 0.2	0.5 ± 0.0 ^b	0.6 ± 0.1 ^a	0.7 ± 0.0 ^a	0.6 ± 0.0 ^a
18:2n-6	3.7 ± 0.8	1.2 ± 0.3 ^b	3.2 ± 0.2 ^a	3.0 ± 0.3 ^a	2.3 ± 0.5 ^{ab}
20:4n-6	1.4 ± 0.1	1.7 ± 0.1 ^{ab}	2.2 ± 0.1 ^a	2.0 ± 0.1 ^{ab}	1.6 ± 0.1 ^b
Other n-6	0.7 ± 0.2	0.6 ± 0.1 ^c	1.7 ± 0.0 ^a	1.2 ± 0.1 ^b	0.8 ± 0.0 ^c
18:3n-3	0.5 ± 0.1	0.1 ± 0.1 ^b	0.6 ± 0.0 ^a	0.6 ± 0.1 ^a	0.3 ± 0.1 ^b
18:4n-3	1.6 ± 0.4	0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
20:4n-3	0.7 ± 0.2	0.4 ± 0.1 ^a	0.2 ± 0.0 ^b	0.3 ± 0.3 ^{ab}	0.2 ± 0.0 ^b
20:5n-3; EPA	7.0 ± 1.1	5.5 ± 0.5 ^a	1.8 ± 0.2 ^b	3.2 ± 0.3 ^b	3.6 ± 0.6 ^{ab}
22:5n-3	3.0 ± 0.5	1.9 ± 0.1 ^a	0.6 ± 0.1 ^c	1.1 ± 0.1 ^{bc}	1.2 ± 0.0 ^b
22:6n-3; DHA	15.5 ± 0.7	11.2 ± 0.5	12.0 ± 0.6	12.5 ± 1.1	10.8 ± 1.0
Other n-3	1.2 ± 0.4	0.5 ± 0.1	0.1 ± 0.0	0.3 ± 0.2	0.3 ± 0.0
Other PUFA	1.5 ± 0.6	0.4 ± 0.3	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.1
Total SFA	32.4 ± 3.7	14.5 ± 1.9	11.9 ± 1.0	13.0 ± 1.8	12.5 ± 1.8
Total MUFA	34.6 ± 6.6	13.4 ± 2.2	16.7 ± 0.6	17.1 ± 1.3	15.7 ± 1.6
Total PUFA	36.8 ± 4.7	24.0 ± 2.0	22.5 ± 1.3	24.5 ± 1.7	21.9 ± 2.7
Total n-3	29.6 ± 3.2	20.1 ± 1.4	15.5 ± 1.0	18.2 ± 1.6	16.8 ± 2.0
Total n-6	5.7 ± 1.0	3.5 ± 0.3 ^c	7.0 ± 0.3 ^a	6.2 ± 0.3 ^{ab}	4.7 ± 0.3 ^{bc}
n-3 LC-PUFA	27.4 ± 2.7	19.5 ± 1.2	14.7 ± 0.9	17.3 ± 1.5	16.2 ± 1.8
DHA: EPA	2.2 ± 0.1	2.1 ± 0.1 ^d	6.6 ± 0.4 ^a	4.0 ± 0.3 ^b	3.0 ± 0.2 ^c
n-3:n-6	5.3 ± 0.5	5.8 ± 0.2 ^a	2.2 ± 0.1 ^c	2.9 ± 0.2 ^b	3.5 ± 0.0 ^b

Values are means ± SEM, $n = 3$. Means in a row sharing different superscript letters were significantly different ($P < 0.05$). FO, 100% fish oil (Jack mackerel); MO 1, a blend of 50% rapeseed and 50% tuna oils; MO 2, a blend of 50% rapeseed, 25% tuna and 25% FO (Jack mackerel); FO/CF, a blend of 50% FO (Jack mackerel) and 50% chicken fat.

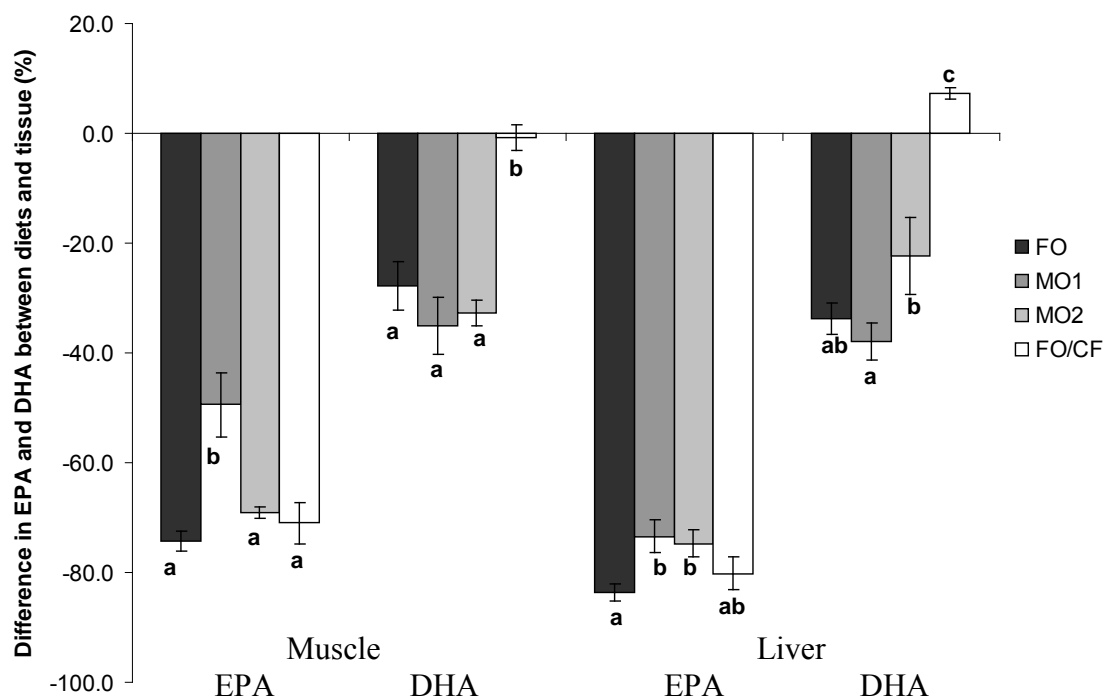


Figure 6.1: Difference in dietary and tissue (muscle and liver) EPA and DHA concentrations – expressed as % difference in dietary and tissue EPA and DHA amounts. Values are means \pm SEM, $n = 3$. Different letters represent significant differences ($P < 0.05$) between treatments for either EPA or DHA in the muscle and liver. FO, 100% fish oil (Jack mackerel); MO 1, a blend of 50% rapeseed and 50% tuna oils; MO 2, a blend of 50% rapeseed, 25% tuna and 25% FO (Jack mackerel); FO/CF, a blend of 50% FO (Jack mackerel) and 50% chicken fat.

6.5 DISCUSSION

In studies involving FO substitution in aquafeeds, the growth performance of fish is probably as important as the FA profile of the resultant seafood products. In the present study, substituting 50% of FO as added oils either with rapeseed oil in MO 1 and MO 2 diets or with chicken fat in FO/CF diet did not affect fish growth. This result is in agreement with previous studies for Atlantic salmon where FO was substituted at 50% or more by different AO (Miller et al., 2008a; Turchini et al.,

2009). The present study was conducted with Atlantic salmon smolt over a period of 75 days, with the aim to test the effect of high dietary DHA: EPA ratios and low dietary n-3 LC-PUFA content on n-3 LC-PUFA deposition in fish tissues. To our knowledge no similar studies have been undertaken and, given the results of this study, it would be of interest to pursue further research to determine the optimum relative and absolute dietary EPA and DHA concentrations which optimize n-3 LC-PUFA deposition in farmed fish.

A major constraint of substituting FO in aquafeeds for Atlantic salmon is the decrease in n-3 LC-PUFA content in fish, therefore a blend of FO and AO in aquafeeds is now common practice. Previous studies have shown that inclusion levels in excess of 25-50% of various vegetable oils (VO) in diets causes a reduction in n-3 LC-PUFA content in Atlantic salmon as compared to fish fed on FO (Bell et al., 2001, 2002, Bransden et al., 2003, Menoyo et al., 2005, 2007). The wide variation in inclusion level partly relate to differences in ingredients such as in the FA composition of the AO used and/or the source FO, in which n-3 LC-PUFA may vary. Differences in experimental design such as feeding regime may also have effects. The n-3 LC-PUFA requirement for Atlantic salmon has been well documented and even with complete substitution of FO in the diet, the requirements can be met from sufficient dietary fishmeal inclusion (Turchini et al., 2009; Tocher et al., 2010). Hence, the major role of FO inclusion in aquafeeds is to convey an excellent source of n-3 LC-PUFA to humans through fish consumption. Presently FO is the only major available source of n-3 LC-PUFA that is commercially viable for use in aquafeeds. In an era of finite resources, it is imperative to judiciously utilize FO (Naylor et al., 2009). In this study, n-3 LC-PUFA content in FO diet was approximately two-fold

higher than for the other diets, but the n-3 LC-PUFA content in the muscle for MO 1 and FO/CF fish was not significantly different to that of FO fish, though higher values were obtained for FO fish compared to the other 3 treatments. This observation indicates that n-3 LC-PUFA, particularly EPA was extensively used for energy production in FO fish. This result is in agreement with other studies on the lipid metabolism of Atlantic salmon (Stubhaug et al., 2007; Chapter 2 and Codabaccus et al., 2011). Feeding a 100% FO diet is regarded as an inefficient practice due to loss by β -oxidation of substantial amounts of the nutritionally valuable n-3 LC-PUFA. In contrast, the oil blends in MO 1 and FO/CF diets provided sufficient balance in dietary FA composition which allowed for more efficient deposition of n-3 LC-PUFA in the muscle. The n-3 LC-PUFA content in the liver was not different among all treatments, reflecting the more important nature of this organ for lipid metabolism particularly that of n-3 LC-PUFA biosynthesis.

When FO is partially substituted by AO in fish diets, it is possible to mimic the FA classes of a FO diet, however, as highlighted earlier, the ratio of DHA: EPA in the diet will remain similar to that of a FO diet. Limited attention has been given to DHA: EPA ratio in diets for Atlantic salmon. It is common to observe a higher DHA: EPA ratio in fish tissues to that of the diet in fish lipid nutrition studies; this results from β -oxidation of surplus dietary EPA and the rather conservative nature of DHA (Brandsen et al., 2003; Miller et al., 2008b; Chapter 2 and Codabaccus et al., 2011). The present study is no exception; in both the muscle and the liver there was an increase in the DHA: EPA ratio in all treatments compared to the ratio observed in their respective diets. The increase in the DHA: EPA ratio was less pronounced with the MO 1 diet due to lower dietary EPA content which reduced the amount of EPA

available for β -oxidation. Besides the EPA and DHA ratio, equally important was their absolute concentration in the diet. Diets can be varied to maintain both EPA at concentrations that minimize β -oxidation and DHA at concentrations for maximum deposition. It has been previously shown that DHA also can be readily used for energy production (Stubhaug et al., 2007; Chapter 2 and Codabaccus et al., 2011); therefore it should not be supplied in dietary surplus. In this study, the difference in dietary and tissue DHA content for all treatments was lower than the difference in EPA content between diet and tissue. This indicates that DHA was preferentially deposited relative to EPA especially with regards to the MO 1 and MO 2 treatments where dietary DHA was higher than dietary EPA. The significance of a proper balance of dietary DHA: EPA ratio and absolute amounts is the n-3 LC-PUFA “saving” effect as observed for MO 1 treatment which had the highest dietary DHA: EPA ratio and the lowest dietary n-3 LC-PUFA content and muscle n-3 LC-PUFA was not significantly different to FO treatment. The difference in dietary and tissue DHA content for the FO/CF treatment followed a different pattern to the other treatments; tissue DHA content mirrored dietary DHA content. The dietary DHA content for the FO/CF treatment was lower than for the other treatments and the fact that there was little difference between dietary and tissue DHA content suggest that the DHA dietary concentration in the FO/CF treatment was more efficient for DHA deposition compared to the other treatments.

In the present study n-3 LC-PUFA deposition in fish tissues was compared with fish fed different dietary concentrations and ratios of EPA and DHA. A difference between relative and absolute dietary EPA and DHA concentrations was used to explain EPA and DHA deposition in muscle and liver. Though it is possible

that some differences between dietary EPA and DHA contents and their respective contents in tissues may be explained by the bioconversion of EPA to DHA, this bioconversion is regarded as negligible, especially in seawater to have a significant impact (Chapter 2 and Codabaccus et al., 2011). This is a simple and direct approach which focuses principally on dietary n-3 LC-PUFA deposition in contrast to studies on complex anabolic and catabolic pathways in fish n-3 LC-PUFA metabolism. The present study has shown the importance of both the relative and absolute concentrations of dietary EPA and DHA for efficient n-3 LC-PUFA deposition in fish tissues by “saving n-3 LC-PUFA” otherwise prone to β -oxidation. A future driving force to pursue research in this direction will ultimately be dependent on progress in the field of plant genomics, particularly genetic engineering of crops producing oil having the desired relative and absolute concentration of EPA and DHA.

In the present context and from an industry perspective, the FO/CF diet is a useful current option because FO/CF fed fish had comparable n-3 LC-PUFA content to FO fish, and importantly oils from rendered animals are less expensive than both FO and VO (Turchini et al., 2009). In addition, the n-6 FA content in FO/CF fish was lower than that of MO 1 and MO 2 fish due to lower dietary n-6 FA content, thus resulting in a higher n-3: n-6 ratio in FO/CF fish than in MO 1 and MO 2 fish. Therefore, from a human health aspect, the FO/CF fish would be more advantageous than MO 1 and MO 2 fish. As well as increasing n-3 LC-PUFA content in plants, research in the genetic engineering of crops producing oils is also aiming at reducing the n-6 FA. Such oils are likely to be available in the future and aquaculture feeding trials for Atlantic salmon and other key species would be very informative for

optimizing the composition of these novel land plant derived oils containing EPA and DHA

6.6 CONCLUSION

A major outcome of this study was that a higher DHA: EPA ratio than that commonly experienced with FO-based diets for Atlantic salmon is better suited for efficient deposition of n-3 LC-PUFA. Future research will be needed to determine the optimum relative and absolute concentrations of dietary EPA and DHA to maximize n-3 LC-PUFA deposition in Atlantic salmon. The rationale to pursue such studies will be reliant on research in plant genomics since oils with the desired FA characteristics are not presently commercially available. The use of a 50% FO and 50% CF blend in aquafeeds for Atlantic salmon, as is now commercially practised in Tasmania, resulted in comparable n-3 LC-PUFA content in the muscle and liver of juvenile Atlantic salmon to a FO fish. Such an oil blend decreases the inefficient utilization of a 100% FO diet due to the high loss of EPA in particular and may be considered as an appropriate strategy for use in aquafeeds for Atlantic salmon.

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CHAPTER 7

General Discussion

7.1 *Overview*

This thesis examines current and innovative strategies to maintain n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid (LC-PUFA) levels in salmonids fed diets where fish oil (FO) is substituted, with emphasis on the use of novel lipid sources. A common strategy that was investigated in this thesis was feeding fish with a diet comprising a blend of alternative oil (AO) and FO, where FO was substituted at medium levels (typically 50%). Another strategy examined consisting of a two step process in which fish are fed primarily a grow-out diet where FO is substituted at medium to high levels ($\geq 50\%$) followed by a feeding period with a FO finishing diet (FOFD). Innovative strategies include the maximization of endogenous n-3 LC-PUFA capacity of fish by using a stearidonic acid (SDA, 18:4n-3) rich diet as a precursor for n-3 LC-PUFA biosynthesis, reduction of lipid content in fish fillet after growth on AO-based diet prior to feeding a FOFD, and the novel concept of n-3 LC-PUFA “saving” by use of a high dietary DHA: EPA ratio coupled with lower n-3 LC-PUFA content to that usually encountered with a typical FO-based diet. These strategies were investigated in the research Chapters 2-6 and the following discussions relate to the major findings and their application as a strategy to maintain n-3 LC-PUFA levels particularly for Atlantic salmon.

7.2 *Maximizing n-3 LC-PUFA endogenous biosynthetic capacity*

Chapters 2 and 3 focus on the n-3 LC-PUFA endogenous biosynthetic capacity of Atlantic salmon for whole body (organism), liver (organ) and muscle (tissue). The bioconversion of n-3 LC-PUFA from α -linolenic acid (ALA) proceeds through a series of desaturase and elongase enzymes. The bioconversion of ALA to SDA which involves a $\Delta 6$ desaturase is a rate limiting step (Brenner, 1981). *Echium*

oil (EO) is naturally rich in SDA (~ 14%) and its position along the n-3 biosynthetic pathway may cause this rate limiting $\Delta 6$ desaturase to be bypassed through product feedback inhibition by supply of SDA. Consequently, n-3 LC-PUFA biosynthesis may be maximized. This hypothesis has been confirmed for the whole body (Chapter 2) using a fatty acid mass balance (FAMB) approach and for liver and muscle (Chapter 3) by measuring gene expression of n-3 LC-PUFA biosynthetic enzymes for Atlantic salmon in freshwater and seawater. Higher n-3 LC-PUFA was obtained for EO fed fish compared to fish fed on rapeseed oil (RO)-based diet. A synthesis of results obtained for the whole body, liver and muscle depicts the differential n-3 LC-PUFA biosynthetic capacity among these three levels and the influence of environment on n-3 LC-PUFA biosynthesis. There appears to be an order of n-3 LC-PUFA biosynthesis/deposition with - liver > muscle > whole body, and which reflects their respective biological roles; the liver as the main organ for n-3 LC-PUFA biosynthesis, the muscle containing a high proportion of polar lipids (PL) as structural components of cell membranes which are rich in n-3 LC-PUFA and the whole body principally dominated by storage lipid mainly as triacylglycerols (TAG), and thus more influenced by dietary lipid. It has been well documented that Atlantic salmon in freshwater, particularly when undergoing smoltification, possess higher n-3 LC-PUFA biosynthetic capacity than when they are in seawater (Bell et al., 1997; Tocher et al., 2000; 2003). Similarly for the whole body, a FAMB showed higher n-3 LC-PUFA biosynthesis in freshwater compared to seawater. In the liver, $\Delta 5$ desaturase gene expression was lower in seawater compared to freshwater irrespective of dietary treatment. This is an unprecedented and major finding which may explain the environmental differences in n-3 LC-PUFA biosynthesis in Atlantic salmon.

A key question that remains to be answered is whether the maximization of n-3 LC-PUFA biosynthesis is a suitable strategy for maintaining n-3 LC-PUFA levels in Atlantic salmon? Despite the fact that n-3 LC-PUFA levels were higher in EO fish for whole body, liver and muscle, compared to RO fish, it was lower compared to FO fish. Besides maintaining n-3 LC-PUFA levels, AO should also be less expensive than FO and this undermines the use of EO as an AO since its production is not presently commercially viable. However, in the near future SDA rich oils may become commercially available from genetically modified oilseed plants. It may be argued that such oils would be a better AO to conventional vegetable oils (VO) as a substitute for FO in aquafeeds from a consumer point of view due to higher n-3 LC-PUFA biosynthesis, high SDA and high n-3 PUFA than in VO fed fish. A key point when using this strategy is the absence or strict minimum dietary inclusion of n-3 LC-PUFA so as to not to inhibit endogenous n-3 LC-PUFA biosynthesis. As such this strategy cannot be used in conjunction with a blended approach where a mix of AO and FO in diets is practiced. Since Atlantic salmon in freshwater have the ability to endogenously biosynthesize sufficient amounts of n-3 LC-PUFA to meet their requirements, such a strategy may be beneficial during the freshwater phase of Atlantic salmon farming but not for the entire production period. The advantages of using such a strategy are that no dietary FO is required as well as feeding fish with VO diets promotes smoltification. Thus using this strategy, a significant amount of FO may be reduced during one quarter of the whole production cycle until seawater transfer.

7.3 *Fish oil finishing diet*

The FOFD is a strategy to restore n-3 LC-PUFA levels, particularly that of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) in fish after a grow-out period with an AO-based diet. The FA changes that occur after dietary change are principally a dilution of existing FA stores from the previous diet. A grow-out diet rich in saturated FA (SFA) and/or monounsaturated FA (MUFA) is considered more appropriate since it may favour preferential FA metabolism of existing FA stores from the grow-out diet over new FA, in particular the n-3 LC-PUFA from the FOFD. Thus it may accelerate n-3 LC-PUFA restoration. Palm fatty acid distillate (PFAD) is a cheap novel FA source, is rich in SFA, and therefore is a good candidate to test for use as an AO in the grow-out diet and for the occurrence of preferential FA metabolism when feeding a FOFD. Chapter 4 examines the restoration of EPA and DHA in rainbow trout in freshwater using a FOFD where 50% PFAD and 75% PFAD replaced FO in grow-out diets at optimal (15°C) and elevated (20°C) water temperatures. Rainbow trout was chosen as the test species due to the logistic difficulties with growing harvest size Atlantic salmon in a recirculating system and also several studies using this strategy have been previously undertaken with Atlantic salmon. The use of PFAD as an AO had not yet been investigated for salmonids nor has the effect of temperature on EPA and DHA restoration using the FOFD prior to this study. The results suggested that FA changes that occur after dietary change (from grow-out diet to FOFD) were the result of a dilution of existing FA stores from the previous diet at both optimal and elevated water temperatures, and there was no evidence for FA preferential metabolism occurring. The restoration of EPA and DHA (as % total FA) in rainbow trout fillet and whole carcass of fish previously fed the 50% PFAD diet ranged from 85 to 98% of that in fish fed FO diet

throughout. For fish fed 75% PFAD, restoration of EPA and DHA was lower (from 66 to 93%) in fillet and whole carcass of fish compared to fish fed FO diet throughout. Growing rainbow trout with diets where 50% and 75% FO was replaced by PFAD followed by use of a FOFD, reduced the use of FO by an estimated average of 30 and 44% respectively, at 15°C and 32 and 48% respectively at 20°C. At the highest FO substitution (75% PFAD), during the entire growth period more than 50% FO was used. FOFD diet strategies should be considered simply as a palliative solution to the problem of FO replacement in aquafeeds because complete restoration of optimal FA composition cannot be achieved and in attempting to do so a significant amount of FO is still required (Turchini et al., 2009). Therefore for the short to medium term the FOFD strategy is suitable to maintain n-3 LC-PUFA levels in fish until new strategies are achieved that further reduce the FO input in aquafeeds concomitant with maintaining n-3 LC-PUFA levels in fish. Growth was impaired for fish grown with 75% PFAD diets during the grow-out period. The reasons for this observation are unclear since the apparent digestibility of SFA was improved which results in higher energy availability (Appendix and Ng et al., 2010). Consequently, further research is required to investigate fish growth at high PFAD inclusion levels in diets for rainbow trout.

7.4 *Reduction of fillet lipid content prior to feeding a fish oil finishing diet*

The use of the FOFD strategy to restore n-3 LC-PUFA levels in fish after a grow-out period on AO-based diet is principally a dilution of existing FA stores from the grow-out diet. Consequently, a significant amount of FO is still required in aquafeeds to completely restore n-3 LC-PUFA levels when using this strategy (Chapter 4; Turchini et al., 2009). One way to improve the restoration of n-3 LC-

PUFA levels in the fish fillet is to reduce the initial lipid content in the fillet after growth on the AO-based diet and prior to feeding the FOFD (Palmeri et al., 2009). In this context, a short term food deprivation may be applied. This strategy was investigated in Atlantic salmon smolt fed a 75% PFAD grow-out diet (75PFAD) for 77 days followed by a short term food deprivation of 7 days prior to feeding a FOFD for 21 days (Chapter 5). This study was conceptual due to the logistical problem of growing Atlantic salmon to harvest size in a recirculated system. The results showed that a 7 day food deprivation period significantly reduced lipid content in fish fillet; after 21 days of the FOFD period, the n-3 LC-PUFA % composition was higher than in the fillet of fish not deprived of feed and fed for 4 weeks a FOFD. The absolute amount of n-3 LC-PUFA in fish fillet was not significantly different between the two treatment groups, although higher values were obtained for fish unfed for 7 days followed by 21 days of feeding with FOFD. The relative levels and absolute amounts of n-3 LC-PUFA in the fillet of fish fed 75PFAD for 77 days followed by 28 days FOFD was restored to 72% and 71% respectively, of that of fish fed FO throughout. The relative levels and absolute amounts of n-3 LC-PUFA in the fillet of fish fed 75PFAD for 77 days followed by 7 days food deprivation then 21 days FOFD were restored to 81% and 80% respectively, of that of fish fed FO throughout. The significance of this study is that there was a reduction in the FOFD period, thus a reduction in dietary FO input and a higher degree of n-3 LC-PUFA restoration. The likely principle is that by reducing the initial lipid content in the fillet by food deprivation after grow-out on the AO oil diet, there is a reduction in lipid stores accumulated from AO oil, mainly as TAG and which are low in n-3 LC-PUFA. The resulting fillet will then contain higher relative levels of PL (rich in n-3 LC-PUFA) and by subsequent feeding with FOFD, the dilution of existing FA stores will be to a

lesser extent compared to a typical FOFD strategy and therefore n-3 LC-PUFA will be more concentrated. It is to be highlighted that this strategy may not be applicable to all fish species, since different fish species may lose lipid in different way and/or at times even use protein for energy production as a result of food deprivation (Palmeri et al., 2009). The key tissue of interest to consumers is the fillet and upon food deprivation it is essential that there is significant loss of fat in the fillet as opposed to other tissues or organs such as the liver or the viscera.

This study was performed with Atlantic salmon of initial weight ~70g at a temperature of 15°C. A key element of this strategy as highlighted above is the reduction of fillet lipid content after growth with a grow-out diet prior to feeding the FOFD. Both the temperature and fish size were favourable for fillet lipid reduction. In larger Atlantic salmon nearing harvest size, a longer period of feed deprivation will likely be needed to significantly reduce fillet lipid content. This may be a setback for using this strategy. During food deprivation, reduction of fillet lipid content increases with temperature (Cho and Bureau, 1995), therefore a food deprivation period may be feasible during summer months. This strategy is a new concept with promising outcomes achieved in terms of favourable manipulation of the n-3 LC-PUFA profile. The next steps would include repeating the trial in the real context of Atlantic salmon farming, with larger fish used.

Unlike with rainbow trout (Chapter 4), there was no difference in growth between Atlantic salmon smolt fed the 75% PFAD and FO diets. Similarly with rainbow trout (Ng. et al., 2010), SFA apparent digestibility was improved for fish fed 75% PFAD due to high content of free fatty acids in PFAD (~ 80%).

7.5 “Saving” *n*-3 LC-PUFA

The concept of *n*-3 LC-PUFA “saving” is a new approach which principally stems from an understanding of lipid metabolism in Atlantic salmon. Surplus dietary EPA in particular is extensively used for β -oxidation (Chapter 2; Stubhaug et al., 2007), DHA is mostly conserved irrespective of its dietary concentration and SFA and/or MUFA are preferentially used for β -oxidation. These observations suggest that by carefully balancing the different FA classes in diets a more efficient deposition of *n*-3 LC-PUFA can be achieved. A strategy of using a blended oil mix may be considered as having similar objectives by reducing dietary *n*-3 LC-PUFA content and still aiming to achieve high *n*-3 LC-PUFA content in fish fed such diets. However, FO is the only commercially available *n*-3 LC-PUFA source and EPA is proportionally higher than DHA (~1.5), therefore any blend of FO and AO will result in the same DHA: EPA ratio (~0.7) with EPA likely to be in surplus. An improved scenario would involve a higher dietary DHA: EPA ratio. This hypothesis was tested in Chapter 6 by comparing *n*-3 LC-PUFA deposition in the liver and muscle of Atlantic salmon fed diets having high DHA: EPA ratios (2.8 and 1.3) and relatively low *n*-3 LC-PUFA content compared to a typical FO diet having low DHA: EPA ratio (0.5) and relatively high *n*-3 LC-PUFA content. A nother treatment of a commercially practised blend comprising a 50:50 mix of FO and chicken fat (CF) was also compared. There was no significant difference in *n*-3 LC-PUFA content in the liver and muscle between the treatments fed diet having the highest DHA: EPA ratio (2.8) and the lowest *n*-3 LC-PUFA content and those fed either FO diet or FO/CF diet. These findings suggest that by the balancing of different FA classes and using a high DHA: EPA ratio, it is possible to “save” *n*-3 LC-PUFA from β -oxidation, thereby enabling their more efficient deposition in fish. To obtain a high dietary

DHA: EPA ratio, purified tuna oil used in human nutraceutical and food products and which is naturally rich in DHA was trialled since presently no such oil is commercially available. However, in the field of plant genomics, a high DHA: EPA ratio has been demonstrated in model plants producing oil (Petrie et al., 2010). Therefore future research aiming at optimising the deposition of n-3 LC-PUFA in fish by the balancing of FA classes and use of a higher DHA: EPA ratio will be pertinent for scientists in the field of plant genomics. Future research on this concept of n-3 LC-PUFA “saving” combining fish culture trials and plant genomics may lead to an oil tailor-made for use in aquafeeds. In the present context, a blended oil approach is considered useful to maintain n-3 LC-PUFA content in fish as observed in the FO/CF treatment.

7.6 *The way forward*

With respect to the findings of this thesis there exist several avenues to improve on existing strategies to maintain n-3 LC-PUFA levels in salmonids. The best practice would likely be not to adopt a singular approach but to integrate the different strategies/concepts investigated within this study taking into consideration the life cycle with respect to culture environments. For example with Atlantic salmon, the maximizing of n-3 LC-PUFA endogenous biosynthetic capacity strategy could be used during the freshwater phase of culture (no FO input) until seawater transfer. After the transfer of fish to seawater, either feeding on a grow-out diet with an AO-based feed followed by a period of food deprivation then a FOFD, or using the n-3 LC-PUFA “saving” strategy could be combined to improve fillet quality in terms of n-3 LC-PUFA content. All these scenarios show considerable promise and should be further validated with Atlantic salmon during an entire production cycle. This

present research focuses on the nutritional aspect of n-3 LC-PUFA deposition in salmonids. Another approach is by selective breeding between families which have high flesh n-3 LC-PUFA levels since it was found that flesh n-3 LC-PUFA composition is a highly heritable trait ($h^2 = 0.77$) (Leaver et al., 2011).

FO substitution in aquafeeds for four major cultured fish species in Europe including Atlantic salmon and rainbow trout has been subject to various studies especially under the European project “Researching alternatives to fish oils in aquaculture, RAFOA” from 2001 to 2005. In conclusion, this European research project showed that significant amounts of FO can be substituted in aquafeeds by VO for salmonids, however the main constraint is the low content of n-3 LC-PUFA in fish with high VO inclusions in diets. Thus, complete substitution of FO is not possible. This thesis has brought significant addition to research in lipid metabolism for salmonids, particularly for Atlantic salmon as well as identifying new and/or improving current strategies to maintain n-3 LC-PUFA. However, FO inclusion in aquafeeds for salmonids will still be required to provide the health benefitting n-3 LC-PUFA to human consumers. The limited supply of wild caught fish is a concern for FO supply, but at the same time fishmeal (FM) supply is also a major issue. Consequently, FM substitution in aquafeeds is also an industry priority for aquaculture. Since studies of FO and FM substitution were carried out independently, it was far from clear to which extent both FO and FM can be replaced in aquafeeds. Thus, another European project, AQUAMAX was initiated, with the main objective to develop alternative fish feeds to replace FO and FM. As aquaculture is expected to continue to grow due to the limited availability of wild fish catch concomitant with an increasing world population, in the long term, another source of n-3 LC-PUFA should

be sought. In this respect, future GM oils, rich in n-3 LC-PUFA may present the best next alternative.

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Appendix

**Ng, W.K., Codabaccus, M.B., Carter, C.G., Nichols, P.D., 2010.
Replacing dietary fish oil with palm fatty acid distillate improves
fatty acid utilization in rainbow trout, *Oncorhynchus mykiss*,
maintained at optimal or elevated water temperature. Aquaculture
309, 165-172**



Replacing dietary fish oil with palm fatty acid distillate improves fatty acid digestibility in rainbow trout, *Oncorhynchus mykiss*, maintained at optimal or elevated water temperature

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ABSTRACT

An experiment was conducted to evaluate the interactive effects of dietary palm fatty acid distillate (PFAD) and water temperature on lipid and fatty acid digestibility in rainbow trout. Three isolipidic diets with 0, 10 or 15% (w/w) PFAD, at the expense of fish oil, were formulated and fed to triplicate groups of trout maintained at water temperatures of 15 or 20 °C. The apparent digestibility (AD) of fatty acids was measured using yttrium oxide as an inert marker. Increasing dietary PFAD, which contains mainly free fatty acids (FFA), led to a significant ($P < 0.05$) improvement in the AD of saturated fatty acids (SFA) but not that of total monounsaturated or polyunsaturated fatty acids in trout maintained at both water temperatures. High AD of total lipids (90–93%) was observed in all treatments. Based on fecal lipid class and fatty acid composition, this improvement in SFA digestibility was due in part to the increased absorption of the FFA as it bypasses the need for lipolysis. Elevated water temperature tended to cause a reduction in the AD of most fatty acids at each corresponding dietary treatment. The AD of individual fatty acids within each water temperature regimen generally decreased with increasing fatty acid chain length and increased with increasing unsaturation, irrespective of diet. In general, no significant interaction between diet and temperature effects on fatty acid digestibility was found.

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1. Introduction

The farming of salmonid fishes such as the Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) account for about 56% of the total fish oil use in commercially manufactured aquafeeds (Tacon and Metian, 2008). Fish oil is produced from small marine pelagic fish and represents a finite fishery resource. The rapid increase in global aquafeed production has resulted in decreasing market availability and increasing market cost of fish oil. The imminent inability of global fish oil supply to support the sustained expansion of aquaculture production has generated much research in evaluating alternatives to fish oil, especially in the diets of salmonids (Torstensen et al., 2000; Rosenlund et al., 2001; Caballero et al., 2002; Carter et al., 2003a; Turchini et al., 2009).

Palm oil is a potential substitute for fish oil in aquafeeds and has been evaluated in many fish species of commercial value (Ng and Gibon, 2010), including in the feeds for Atlantic salmon (Torstensen

et al., 2000; Bell et al., 2002; Ng et al., 2004a; Ng et al., 2007) and rainbow trout (Ng et al., 2003b; Fonseca-Madrigal et al., 2005; Oo et al., 2007). Palm oil is the generic name given to various palm oil products and by-products at different stages of refining and fractionation with each fraction having different physical, chemical and nutritional properties. Research into the use of crude palm oil (CPO) in the diets of salmonids had reported growth and feed utilization efficiency comparable to fish fed equivalent levels of dietary fish oils (Ng et al., 2007). Nevertheless, the high content of saturated fatty acids (SFA) (about 50%) in CPO has been shown to be a significant factor affecting fatty acid digestibility and subsequent energy availability in these cold water fish species (Ng et al., 2003b; 2004a). Replacement of dietary fish oil with increasing levels of CPO was found to markedly reduce the apparent digestibility (AD) of SFA in rainbow trout (Ng et al., 2003b). The overall AD of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) was also negatively affected by increasing CPO levels. Feces collected from trout fed diets with increasing CPO levels contain more than 60% SFA in the non-absorbed fecal lipids and has increasing concentrations of undigested triacylglycerols (TAG). Ng et al. (2003b) concluded that the reduction in the AD of SFA was largely due to the increasing resistance of TAG to digestion with increasing dietary CPO. In fish,

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dietary TAG is largely hydrolyzed into free fatty acids (FFA) and glycerol due to the presence of two lipases (Leger, 1985). The first objective of the present study was to investigate whether SFA from a palm oil source can be better digested and absorbed by rainbow trout when supplied in the form of FFA instead of TAG.

Palm fatty acid distillate (PFAD), a by-product from the physical refining of CPO (Ng and Gibon, 2010), contains about 80% FFA and 14.5% acylglycerols (Ong and Choo, 1997) with a fatty acid composition of about 64% SFA, 30% MUFA and 6% PUFA (Ng et al., 2003a). PFAD has been successfully used as a fish oil substitute in the diets of tropical fish (Ng et al., 2004b; Bahurmiz and Ng, 2007). The effects of increasing dietary levels of PFAD on fatty acid digestibility in salmonid diets have not been previously reported. Considering the lower costs of PFAD as compared to CPO, the use of PFAD in salmonid diets warrants investigation.

The influence of water temperature on lipid and fatty acid digestibility in rainbow trout is not conclusive. Austreng et al. (1980) reported no significant differences in lipid and fatty acid digestibility in rainbow trout reared at 3 °C or 11 °C. In contrast, Windell et al. (1978) reported a significant decrease in lipid digestibility in small size (about 19 g) rainbow trout reared in water temperatures of 7 °C compared to fish in water temperatures of 11 °C or 15 °C. However, this reduction in lipid digestibility was not observed in fish of medium (about 207 g) or large (about 586 g) size at the same temperature. Ng et al. (2003b) reported that lowering water temperature significantly reduced SFA digestibility in trout fed CPO-based diets. When water temperature was dropped from 15 to 7 °C, a 25% reduction in lipid digestibility was observed in trout fed diets containing CPO as the only added oil. The second objective of the present study was to investigate if elevated water temperature has any impact on lipid and fatty acid digestibility in trout. Rainbow trout are known to thrive in water temperatures of 13–18 °C (Hardy, 2002) with an optimal culture temperature of around 14–15 °C. Elevated water temperatures of 19–20 °C are now commonly encountered in Tasmanian waters in the warmer months and this may affect nutrient utilization, such as lipids, in farmed salmonids (Miller et al., 2006). Water temperatures are also rising in many other temperate countries where salmonids are farmed. Battisti and Naylor (2009) predicted that in temperate regions, the hottest seasons currently on record will represent the future norm in many of these locations.

The present study was designed to evaluate the interactive effects, if any, of various dietary PFAD levels and water temperature on lipid and fatty acid digestibility in rainbow trout. An accurate knowledge of the apparent fatty acid digestibility will contribute to optimizing the inclusion of this palm oil product in salmonid diets for use throughout the grow-out season.

2. Materials and methods

2.1. Experimental diets

Three isonitrogenous and isolipidic experimental diets were formulated using the same basal ingredients and varying only in their content of PFAD that was used to replace 0, 50 or 75% of added fish oil and labeled 0%PFAD, 50%PFAD and 75%PFAD, respectively (Table 1). PFAD was properly melted in a water bath and thoroughly mixed with the fish oil before the oil mixture was blended with the dry ingredients. The experimental diets contained low fish meal levels (145 g kg⁻¹ diet) with casein, wheat gluten and soybean meal making up the rest of the protein sources. The diets were produced into 3 mm diameter pellets using a California Pellet Mill (CL-2, San Francisco, CA, USA), fan dried and stored at -5 °C until used (Carter et al., 2003a,b). Yttrium oxide was added to the experimental diets (1.0 g kg⁻¹) as an inert marker for calculations of apparent digestibility of lipid and fatty acids. The ingredient formulation and proximate composition of the experimental diets are shown in Table 1.

Table 1

Ingredient and proximate composition (g kg⁻¹ dry matter) of rainbow trout experimental diets.

	Diet		
	0%PFAD	50%PFAD	75%PFAD
<i>Ingredient composition (g kg⁻¹)</i>			
Fish meal ^a	145	145	145
Casein ^b	145	145	145
Wheat gluten ^c	145	145	145
Soybean meal ^d	145	145	145
Fish oil ^a	200	100	50
Palm fatty acid distillate ^e	0	100	150
Pre-gel starch ^f	127	127	127
Vitamin mix ^g	7	7	7
Mineral mix ^h	7	7	7
Stay-C ⁱ	6	6	6
Choline chloride ^j	2	2	2
Sipernat ^k	40	40	40
CMC ^j	10	10	10
Monobasic calcium phosphate ^j	20	20	20
Yttrium oxide ^j	1	1	1
<i>Chemical composition</i>			
Dry matter (g kg ⁻¹)	908.4	923.4	922.5
Crude protein	417.8	417.6	415.9
Crude fat	225.7	230.8	234.0
Ash	101.0	105.4	101.4
Energy (MJ kg ⁻¹)	22.5	22.4	22.7

^a Skretting Australia, Cambridge, Tasmania, Australia.

^b MP Biomedicals Australasia Pty. Ltd., Seven Hills NSW, Australia.

^c Starch Australasia, Lane Cove, NSW, Australia.

^d Hamlet Protein A/S, Horstens, Denmark.

^e Wilmar Edible Oils Ltd., Penang, Malaysia.

^f Penford Limited, Lane Cove, NSW, Australia.

^g Vitamin mix (ASV4) as listed in Carter et al. (2003).

^h Mineral mix (TM4) as listed in Carter et al. (2003).

ⁱ L-Ascorbyl-2-polyposphate (Roche Vitamins Australia, Frenchs Forest, NSW, Australia).

^j Sigma-Aldrich, Castle Hill, NSW, Australia.

^k Degussa, Frankfurt, Germany.

The fatty acid and lipid class composition of the experimental diets is shown in Tables 2 and 3, respectively.

2.2. Experimental procedure

Rainbow trout (all female fish), with a mean initial body weight of about 133 g, were obtained from a local trout hatchery and stocked into two series of 300 L fiberglass tanks at the School of Aquaculture, University of Tasmania. The partial recirculation system and daily maintenance protocols were as previously described by Miller et al. (2006). Over a 2-week period, the water temperature of a series of nine tanks was gradually increased to 20 ± 1 °C, while another series of nine tanks were maintained at a water temperature of 15 ± 1 °C. The indoor tanks were subjected to a photoperiod regime of 16-h light:8-h dark. All fish were fed a commercial trout pellet (Skretting, Tasmania, Australia) during this acclimation period. After two weeks, each group of temperature-acclimatized fish was randomly redistributed into the nine tanks at 30 fish per tank. Fish maintained at each temperature grouping was then fed one of the three experimental diets (three replicate tanks per diet) for six weeks before the commencement of feces collection. Fish were fed twice daily at a fixed ration of 1.5% body weight per day. A fixed feeding rate was used to avoid the potential influence of different feed intake between fish maintained at different water temperature on nutrient digestibility estimates. The amount of feed ration chosen is in accordance with commercial feeding practices for rainbow trout (Hardy, 2002). Fish were batch weighed every two weeks to readjust the feed ration.

After six weeks, surviving fish from each tank were randomly removed for fecal collection. In the morning, about three hours after

Table 2
Fatty acid composition (% total fatty acids) of rainbow trout experimental diets.

Fatty acid	Diet		
	0%PFAD	50%PFAD	75%PFAD
14:0	6.3	3.6	2.4
15:0	0.6	0.3	0.2
16:0	21.3	33.5	39.6
17:0	0.6	0.3	0.2
18:0	4.6	4.6	4.5
20:0	0.2	0.3	0.3
Other SFA ^a	0.8	0.4	0.1
16:1n-7c	8.6	4.4	2.6
18:1n-9c	11.2	23.7	29.3
18:1n-7c	3.4	2.2	1.6
20:1n-9	2.6	1.4	0.7
24:1n-9c	0.6	0.3	0.1
22:1n-11c	2.1	1.0	0.5
Other MUFA ^b	1.8	0.8	0.1
18:2n-6	4.6	8.0	9.2
20:4n-6	1.0	0.4	0.2
16:4n-3	1.2	0.6	0.4
18:3n-3	1.1	0.7	0.6
18:4n-3	1.8	1.0	0.5
20:4n-3	1.0	0.6	0.4
20:5n-3	11.1	5.6	3.0
22:5n-3	1.8	0.8	0.4
22:6n-3	8.2	4.0	2.1
Other PUFA ^c	2.8	1.2	0.4
Total SFA	34.5	43.0	47.4
Total MUFA	30.5	33.9	35.0
Total PUFA	34.9	23.0	17.5
Total n-3	27.5	13.8	7.7
Total n-6	6.2	8.6	9.5
n-3/n-6	4.5	1.6	0.8

^a Saturated fatty acids.^b Monounsaturated fatty acids.^c Polyunsaturated fatty acids.

the last feeding, fish from each tank were immersed in anaesthetic (benzocaine, 100 mg L⁻¹) and fecal samples collected from the hind gut region by gently squeezing the ventral abdominal area (Austreng, 1978). Fecal samples were pooled by tank and stored at -20 °C prior to analysis of lipid content, lipid class, fatty acid composition and yttrium oxide. After fecal stripping, all contributing fish were killed by a blow to the head. The experimental procedure was conducted in accordance with the University of Tasmania Animal Ethics guidelines.

2.3. Chemical analysis

Dry matter of the experimental diets was determined by freeze drying to constant weight. Crude fat was determined according to Bligh and Dyer (1959) and crude protein by Kjeldahl using a selenium catalyst and calculated as N×6.25. Samples were placed in a muffle furnace at 600 °C for combustion to determine ash content. Gross energy was determined using an adiabatic bomb calorimeter (Gallenkamp autobomb). Yttrium oxide (Y₂O₃) was analyzed by an inductively coupled plasma emission spectrophotometer. Dried diet

and feces were digested with 16 M HNO₃ and 30% (w/v) hydrogen peroxide (1:1, by vol) until colorless before Y₂O₃ analysis.

2.4. Lipid class and fatty acid analysis

Total lipid was extracted from freeze-dried diets and pooled fish feces using a modified Bligh and Dyer (1959) protocol. Weighed samples were soaked overnight in CHCl₃/MeOH/H₂O (1:2:0.8, by vol) in a separatory funnel. The next morning, CHCl₃ and H₂O containing NaCl (9 g L⁻¹) was added for a final solvent ratio of 1:1:0.9 (vol) for separation of total lipids. Total lipid content of samples was determined gravimetrically after evaporation of solvents using a rotary evaporator.

Lipid class composition of experimental diets and fish feces was determined according to the procedures described by Miller et al. (2006). Lipid classes were analyzed using an Iatroscan MK TLC-FID analyzer (Iatron Laboratories, Tokyo, Japan). Separation of lipid classes was done by spotting extracted total lipids onto silica gel SIII Chromarods (5 µm particle size) and developing the rods in a glass tank with hexane/diethyl ether/acetic acid (60:17:0.1, by vol). Quantification was performed using FID and of DAPA software (Kalamunda, Western Australia, Australia).

Fatty acid methyl esters (FAME) were prepared by acid-catalyzed transesterification of total lipid as described in Miller et al. (2006). An internal standard (19:0 FAME) was added for quantitative measurement of individual fatty acids present in diets and feces. FAME were resolved and analyzed by gas-liquid chromatography using an Agilent Technologies 7890A gas chromatograph (Palo Alto, CA, USA) equipped with an Equity™-1 fused silica capillary column (15 m×0.1 mm i.d.), an FID, a split/splitless injector and an Agilent Technologies 7683 B Series auto sampler. Helium was used as carrier gas. Samples were injected in splitless mode at an oven temperature of 120 °C. After injection, oven temperature was raised to 270 °C at 10 °C min⁻¹ and finally to 310 °C at 5 °C min⁻¹. FAME peaks were identified by comparing retention time with those of known standards and by mass spectral data. Peaks were quantified using Agilent Technologies GC ChemStation software.

2.5. Digestibility calculations and statistical analysis

The apparent digestibility (% AD) of dietary fatty acids (dry weight basis) was calculated as: $100 - [100 \times (Y_2O_3 \text{ conc. in feed} / Y_2O_3 \text{ conc. in feces}) \times (\text{fatty acid conc. in feces} / \text{fatty acid conc. in feed})]$. The quantitative amount of individual fatty acids in diets and feces were calculated based on the relative proportion of each fatty acid compared to a known amount of internal standard (19:0) added and the total lipid determined in samples. All percentage data were arcsine transformed before statistical analysis. Normality and homogeneity of variance were confirmed before comparison between means was conducted using two-way ANOVA. Multiple comparisons (effects of diet, temperature and interaction) were achieved by Tukey-Kramer HSD and effects were considered significant at $P < 0.05$. All statistical analysis was performed using the SPSS software version 11 (SPSS, IL, USA).

3. Results

3.1. Fatty acid and lipid class composition of diets

The three experimental diets were essentially similar in protein, lipid and energy content (Table 1). However, the fatty acid composition of diets was distinctly different and consistent with the increasing levels of added PFAD at the expense of fish oil (Table 2). The diet with fish oil as the sole lipid source contained the highest and the lowest relative concentrations of n-3 and n-6 PUFA, respectively, compared to other diets. The n-3/n-6 ratios were 4.5, 1.6 and 0.8 for

Table 3
Lipid class composition (% total lipid) of rainbow trout experimental diets.

Lipid fraction	Diet		
	0%PFAD	50%PFAD	75%PFAD
Wax ester	0.3	0.6	0.3
Triacylglycerol	94.4	61.0	32.6
Free fatty acid	0.7	31.5	60.3
Sterols	0.5	0.6	0.2
Polar lipids ^a	4.1	6.3	6.5

^a Polar lipids include phospholipids, fatty acid polymers, cross-linked polar lipid classes and possible pigments.

0%PFAD, 50%PFAD and 75%PFAD diets, respectively. With increasing dietary PFAD, total SFA (in particular, 16:0) and MUFA (in particular, 18:1n-9) levels were increased in the diets. As a percentage of total fatty acids, total SFA increased from 34.5 to 47.4% and total MUFA from 30.5 to 35.0%, but total PUFA decreased from 34.9 to 17.5% with substitution of 0 to 75% of added fish oil with PFAD, respectively.

As expected, there was a drastic decrease in TAG and a corresponding increase in % FFA with increasing PFAD in the trout diets (Table 3). The concentrations of wax ester and sterols remained similar. Polar lipids were about 2% higher in the 75%PFAD diet compared to the control fish oil diet (0%PFAD).

3.2. Lipid class and fatty acid composition of feces

Total lipid in feces was significantly affected ($P < 0.05$) by increasing PFAD in the diets of trout maintained at optimal or elevated water temperatures (Table 4). Fecal lipid content was significantly lower in fish fed PFAD-based diets compared to fish fed the fish oil-based diet. When PFAD was increased from 100 (50%PFAD) to 150 (75%PFAD) g kg⁻¹ diet, a further decrease in fecal lipid content was observed but this difference was not significant. Water temperature did not significantly affect fecal lipid content and there was no interactive effect between diet and temperature.

Lipid class composition of the excreted fecal lipid was markedly different among the three dietary treatments (Table 4). With increasing dietary PFAD, % FFA in fecal lipids was increased with a corresponding decrease in % TAG for both water temperature treatments. The changes in TAG content were more prominent compared to FFA when dietary PFAD was increased from 100 to 150 g kg⁻¹ diet. Wax esters in fecal lipids were significantly increased with increasing dietary PFAD, but these changes and those of sterols and polar lipids were mostly minor. Water temperature did not significantly affect fecal lipid class composition. With the exception of sterols, no interaction between diet and temperature was found for all lipid classes.

Compared to the diets, fecal total SFA concentrations were relatively higher while PUFA were present in much lower amounts (Table 5). Among the PUFA, n-3 fatty acids were relatively more reduced in feces compared to n-6 fatty acids, especially in fish fed the 0%PFAD diet. The proportion of total MUFA was slightly lower in the feces compared to that found in the diets. Fecal fatty acid profiles were very different compared to diets and the differences between the diets were not always maintained in the corresponding fecal samples. For example, the level of 16:0 in the 75%PFAD diet is almost twice the amount found in the 0%PFAD diet (Table 2), but this difference was not observed in the corresponding fecal samples (Table 5) for both water temperatures. Increasing dietary PFAD significantly reduced

the proportion of fecal total SFA content and increased total MUFA content. Fecal total PUFA content was not significantly affected by diet. Water temperature did not significantly impacted the total SFA, MUFA and PUFA of fecal lipids, but some individual fatty acids were significantly affected by the change in water temperature (Table 5). In general, there was no interactive effect between diet and temperature on fecal fatty acid composition (data not shown).

3.3. Apparent digestibility of fatty acids

The AD of individual fatty acids within each water temperature regimen generally decreased with increasing fatty acid chain length and increased with increasing unsaturation irrespective of diet (Table 6). The AD of total PUFA was highest, followed by total MUFA and lastly by total SFA. AD of total n-3 PUFA was consistently higher compared to total n-6 PUFA at each corresponding diet and temperature treatments.

Digestibility of SFA increased with increasing dietary PFAD level (Table 6). With the exception of 22:0, most of these improvements in SFA digestibility were significantly better even at the 50% replacement level of added fish oil with PFAD. For example, in fish maintained at 15 °C, the AD of 16:0 increased from 85.6% (fed 0%PFAD diet) to 94.8% (fed 75%PFAD diet). A similar trend was observed in the AD of 16:0 in fish maintained at the elevated water temperature of 20 °C. The AD of MUFA and PUFA were mostly not significantly affected by dietary treatment. Total fatty acid digestibility was significantly better in fish fed the 75%PFAD diet compared to fish on the 0%PFAD diet for both water temperatures. Increasing dietary PFAD did not reduce total lipid digestibility in rainbow trout reared at 15 °C or 20 °C with AD of total lipids registering above 90% for all diets.

Elevating the water temperature tended to improve 14:0 and 15:0 digestibility in trout regardless of diet. In contrast, AD of most SFA was not significantly affected by water temperature and tended to be slightly higher in fish maintained at 15 °C compared to fish at 20 °C for each corresponding dietary treatment. Similarly, in general, the AD of MUFA and PUFA tended to be slightly higher in fish maintained at their optimal water temperature compared to fish maintained at elevated water temperature. Total lipid and fatty acid digestibility was also slightly higher in fish maintained at 15 °C compared to 20 °C.

Diet significantly affected the AD of all SFA with the exception of 22:0 (Table 7). The AD of 16:1n-7, 22:1n-11 and 24:1n-9 was significantly affected by diet but not the other MUFA. Among the PUFA, AD of 18:3n-3 and 22:6n-3 was significantly affected by dietary treatment. With the exception of total SFA and total fatty acids, the AD of total MUFA, total PUFA and total lipid was not significantly affected by increasing dietary levels of PFAD. Water temperature significantly

Table 4
Total lipid (g kg⁻¹) and lipid class composition (% total lipid) of feces collected from trout fed increasing dietary levels of palm fatty acid distillate (PFAD) as % of added oil and maintained at optimal or elevated water temperature¹.

PFAD (%)	Temp. (°C)	Lipid class ²					Total lipid
		WE	TAG	FFA	ST	PL	
0	15	0.9 ± 0.2 ^a	24.7 ± 3.0 ^c	28.7 ± 1.4 ^a	9.6 ± 0.3 ^b	36.0 ± 2.1	107.6 ± 5.4 ^b
50	15	1.9 ± 0.6 ^a	11.4 ± 1.4 ^b	45.8 ± 2.9 ^b	4.6 ± 0.7 ^a	36.4 ± 0.4	83.7 ± 1.7 ^a
75	15	3.9 ± 0.4 ^b	5.9 ± 0.8 ^a	45.0 ± 1.1 ^b	5.0 ± 0.7 ^a	40.1 ± 2.6	75.2 ± 2.7 ^a
0	20	0.7 ± 0.1 ^A	17.6 ± 1.2 ^C	35.8 ± 1.1 ^A	6.0 ± 0.4 ^A	40.0 ± 0.1	99.7 ± 1.3 ^B
50	20	2.0 ± 0.7 ^A	12.0 ± 3.1 ^B	41.2 ± 2.7 ^B	5.4 ± 1.0 ^A	39.4 ± 3.2	90.7 ± 3.3 ^A
75	20	4.0 ± 0.9 ^B	3.1 ± 0.5 ^A	46.2 ± 4.5 ^B	6.8 ± 0.4 ^B	39.9 ± 3.3	84.1 ± 8.3 ^A
Two-way ANOVA (P) ³							
Diet		<0.05	<0.05	<0.05	<0.05	0.6	<0.05
Temperature		0.94	0.07	0.59	0.52	0.26	0.48
Diet x Temp. interaction		0.96	0.18	0.13	<0.05	0.66	0.16

¹ Values (mean ± SE, n = 3) in the same column within the same water temperature with different superscripts are significantly different ($P < 0.05$).

² Lipid fractions are wax ester (WE), triacylglycerol (TAG), free fatty acid (FFA), sterol (ST) and polar lipids (PL). See Table 3 footnote.

³ Significance probability associated with the F -statistic.

Table 5

Fatty acid composition (% total fatty acids) of feces collected from trout fed increasing dietary levels of palm fatty acid distillate (PFAD) and maintained at optimal or elevated water temperature¹.

Fatty acid	15 °C			20 °C		
	0%PFAD	50%PFAD	75%PFAD	0%PFAD	50%PFAD	75%PFAD
14:0	8.0 ± 0.1 ^{c*}	4.0 ± 0.1 ^{b*}	2.7 ± 0.2 ^{a*}	5.3 ± 0.9 ^C	3.1 ± 0.4 ^B	1.5 ± 0.2 ^A
15:0	1.0 ± 0.0 ^{c*}	0.5 ± 0.0 ^{b*}	0.3 ± 0.0 ^{a*}	0.8 ± 0.0 ^C	0.4 ± 0.0 ^B	0.1 ± 0.1 ^A
16:0	45.7 ± 1.1	47.0 ± 1.1	42.3 ± 2.2	38.2 ± 1.5	47.1 ± 1.4	46.3 ± 5.8
17:0	1.4 ± 0.0 ^{c*}	0.7 ± 0.0 ^{b*}	0.4 ± 0.0 ^{a*}	1.3 ± 0.0 ^C	0.6 ± 0.0 ^B	0.4 ± 0.0 ^A
18:0	13.1 ± 0.5 ^{c*}	9.2 ± 0.2 ^{b*}	7.5 ± 0.3 ^{a*}	12.3 ± 0.6 ^C	8.8 ± 0.2 ^B	7.9 ± 0.7 ^A
20:0	1.1 ± 0.1	4.1 ± 1.7	0.7 ± 0.0	1.1 ± 0.0	0.7 ± 0.0	0.7 ± 0.1
22:0	0.7 ± 0.0 ^b	0.4 ± 0.1 ^a	0.3 ± 0.0 ^a	0.8 ± 0.1 ^B	0.4 ± 0.0 ^A	0.2 ± 0.1 ^A
Other SFA ²	0.9 ± 0.0 ^b	0.4 ± 0.0 ^a	0.3 ± 0.0 ^a	0.9 ± 0.3 ^B	0.3 ± 0.1 ^A	0.1 ± 0.1 ^A
16:1n-7c	2.9 ± 0.2 ^{b*}	1.1 ± 0.2 ^{a*}	1.1 ± 0.0 ^{a*}	3.6 ± 0.2 ^B	1.6 ± 0.0 ^A	1.1 ± 0.3 ^A
18:1n-9c	6.0 ± 0.2 ^a	18.8 ± 1.3 ^b	29.3 ± 1.7 ^c	8.3 ± 0.5 ^A	20.6 ± 1.2 ^B	27.2 ± 4.1 ^C
18:1n-7c	2.5 ± 0.1 ^{bc}	2.2 ± 0.3 ^{ab}	1.7 ± 0.1 ^a	3.2 ± 0.2 ^{BC}	1.9 ± 0.1 ^{AB}	1.7 ± 0.2 ^A
20:1n-9	2.3 ± 0.1 ^{b*}	1.0 ± 0.0 ^{a*}	0.8 ± 0.1 ^{a*}	3.3 ± 0.2 ^B	1.5 ± 0.2 ^A	1.0 ± 0.3 ^A
20:1n-7c	0.4 ± 0.0 ^c	0.3 ± 0.1 ^b	0.1 ± 0.0 ^a	0.5 ± 0.0 ^C	0.2 ± 0.0 ^B	0.0 ± 0.0 ^A
22:1n-11c	2.5 ± 0.1 ^b	1.1 ± 0.1 ^a	0.7 ± 0.0 ^a	3.6 ± 0.3 ^B	1.4 ± 0.1 ^A	0.9 ± 0.1 ^A
24:1n-9c	1.7 ± 0.1 ^b	0.9 ± 0.0 ^a	0.7 ± 0.1 ^a	2.0 ± 0.0 ^B	0.9 ± 0.0 ^A	0.7 ± 0.0 ^A
Other MUFA ³	1.5 ± 0.1 ^b	0.5 ± 0.0 ^a	0.3 ± 0.0 ^a	1.5 ± 0.2 ^B	0.3 ± 0.1 ^A	0.0 ± 0.0 ^A
18:2n-6	2.1 ± 0.0 ^a	4.2 ± 0.3 ^b	7.1 ± 0.6 ^c	2.8 ± 0.1 ^A	5.2 ± 0.4 ^B	6.4 ± 1.1 ^C
18:3n-3	0.4 ± 0.0 ^b	0.1 ± 0.1 ^a	0.2 ± 0.1 ^a	0.4 ± 0.0 ^B	0.2 ± 0.1 ^{AB}	0.0 ± 0.0 ^A
20:5n-3	1.5 ± 0.2 ^{b*}	1.4 ± 0.2 ^{ab*}	1.0 ± 0.1 ^{a*}	2.7 ± 0.5 ^B	1.4 ± 0.1 ^{AB}	1.2 ± 0.3 ^A
22:5n-3	0.5 ± 0.1 ^b	0.4 ± 0.1 ^a	0.3 ± 0.0 ^a	0.8 ± 0.2 ^B	0.2 ± 0.1 ^A	0.2 ± 0.2 ^A
22:6n-3	2.7 ± 0.5 ^{b*}	1.4 ± 0.1 ^{a*}	2.0 ± 0.2 ^{a*}	5.0 ± 0.9 ^B	2.5 ± 0.1 ^A	2.3 ± 0.4 ^A
Other PUFA ⁴	1.2 ± 0.3 ^b	0.2 ± 0.1 ^a	0.1 ± 0.0 ^a	1.6 ± 0.2 ^B	0.4 ± 0.1 ^{AB}	0.1 ± 0.1 ^A
Total SFA	71.9 ± 1.6 ^b	66.2 ± 1.7 ^{ab}	54.6 ± 2.6 ^a	60.7 ± 2.9 ^B	61.6 ± 1.7 ^B	57.2 ± 6.8 ^A
Total MUFA	19.8 ± 0.7 ^a	25.9 ± 1.3 ^a	34.6 ± 1.8 ^b	25.9 ± 1.2 ^A	28.5 ± 1.3 ^A	32.6 ± 4.9 ^B
Total PUFA	8.3 ± 0.9	7.9 ± 0.4	10.7 ± 0.9	13.3 ± 1.9	9.9 ± 0.4	10.2 ± 2.0
Total n-3	5.7 ± 0.9 ^{b*}	3.6 ± 0.1 ^{a*}	3.6 ± 0.4 ^{a*}	10.1 ± 1.8 ^B	4.7 ± 0.2 ^A	3.8 ± 1.0 ^A
Total n-6	2.5 ± 0.1 ^a	4.3 ± 0.4 ^b	7.2 ± 0.5 ^c	3.2 ± 0.1 ^A	5.3 ± 0.3 ^B	6.4 ± 1.1 ^C
n-3/n-6	2.3 ± 0.3 ^b	0.8 ± 0.1 ^a	0.5 ± 0.0 ^a	3.1 ± 0.4 ^B	0.9 ± 0.1 ^A	0.6 ± 0.1 ^A

¹ Values (mean ± SE, n = 3) in the same row within the same water temperature with different superscripts are significantly different ($P < 0.05$). An asterisk (*) denotes significant differences between temperature treatments ($P < 0.05$).

² Includes 15:0, 15:0, 17:0, 21:0, 24:0.

³ Includes 16:1n-9, 16:1n-5, 17:1, 17:1n-8, 18:1n-5, 22:1n-9, 22:1n-7 and 24:1n-7.

⁴ Includes 18:3 n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:2n-6, 22:4n-6, 22:5n-6, 16:3, 16:4n-3, 18:4n-3, 20:4n-3, 21:5n-3, 22:4n-3, 24:5n-3 and 24:6n-3.

Table 6

Apparent digestibility (%) of total lipid and fatty acids in rainbow trout maintained at optimal or elevated water temperature and fed diets supplemented with increasing levels of palm fatty acid distillate (PFAD)¹.

Fatty acid ²	15 °C			20 °C		
	0%PFAD	50%PFAD	75%PFAD	0%PFAD	50%PFAD	75%PFAD
14:0	91.4 ± 0.5 ^{a*}	93.7 ± 0.4 ^{ab*}	94.5 ± 0.9 ^{b*}	93.3 ± 1.4 ^A	94.9 ± 0.9 ^{AB}	96.3 ± 0.8 ^B
15:0	88.7 ± 0.5 ^{a*}	92.0 ± 0.3 ^{a*}	93.1 ± 1.1 ^{b*}	89.6 ± 1.2 ^A	92.4 ± 0.8 ^A	98.5 ± 1.5 ^B
16:0	85.6 ± 0.5 ^a	92.1 ± 0.4 ^b	94.8 ± 0.8 ^b	85.8 ± 1.6 ^A	91.8 ± 0.5 ^B	92.7 ± 1.7 ^B
17:0	82.7 ± 0.5 ^a	89.3 ± 0.6 ^b	91.9 ± 1.2 ^b	81.7 ± 1.9 ^A	89.3 ± 0.3 ^B	90.6 ± 1.0 ^B
18:0	80.8 ± 0.4 ^a	88.6 ± 0.5 ^b	91.9 ± 1.2 ^b	78.5 ± 3.3 ^A	88.8 ± 0.3 ^B	89.1 ± 2.2 ^B
22:0	80.9 ± 0.7	80.8 ± 2.3	87.2 ± 1.7	73.4 ± 5.7	80.7 ± 0.3	86.5 ± 6.8
16:1n-7c	97.7 ± 0.3 ^{a*}	98.6 ± 0.3 ^{ab*}	98.0 ± 0.2 ^{ab*}	96.7 ± 0.4 ^A	97.9 ± 0.0 ^B	97.4 ± 0.8 ^{AB}
18:1n-9c	96.4 ± 0.3 [*]	95.5 ± 0.6 [*]	95.3 ± 0.3 [*]	94.2 ± 0.6	95.0 ± 0.2	94.5 ± 1.0
18:1n-7c	95.0 ± 0.4	94.5 ± 0.7	95.1 ± 0.4	92.6 ± 0.8	95.0 ± 0.2	93.7 ± 1.3
20:1n-9	93.9 ± 0.5 [*]	95.9 ± 0.4 [*]	94.6 ± 0.4 [*]	90.0 ± 0.7	93.8 ± 0.6	91.1 ± 3.3
20:1n-7c	90.6 ± 0.6	84.1 ± 4.0	83.9 ± 1.2	88.2 ± 0.9	90.1 ± 1.2	89.9 ± 10.1
22:1n-11c	91.8 ± 0.8 ^{a*}	93.9 ± 0.5 ^{ab*}	93.1 ± 0.9 ^{ab*}	86.4 ± 1.6 ^A	91.8 ± 0.7 ^B	89.2 ± 2.5 ^{AB}
24:1n-9c	82.0 ± 1.0 ^{ab}	84.0 ± 0.9 ^b	78.0 ± 4.4 ^a	75.2 ± 2.5 ^{AB}	82.6 ± 0.3 ^B	73.5 ± 4.0 ^A
18:2n-6	97.0 ± 0.1 [*]	97.0 ± 0.4 [*]	96.4 ± 0.1 [*]	95.3 ± 0.5	96.2 ± 0.1	95.8 ± 0.9
18:3n-3	97.6 ± 0.1 ^a	99.1 ± 0.9 ^b	98.4 ± 0.8 ^b	97.2 ± 0.5 ^A	98.4 ± 0.8 ^B	100.0 ± 0.0 ^B
20:5n-3	99.1 ± 0.2 [*]	98.6 ± 0.2 [*]	98.5 ± 0.1 [*]	98.0 ± 0.5	98.5 ± 0.1	97.6 ± 0.8
22:5n-3	98.2 ± 0.3	97.2 ± 0.3	95.8 ± 0.5	96.3 ± 0.8	98.6 ± 0.7	96.6 ± 3.4
22:6n-3	97.8 ± 0.5 ^{ab*}	98.0 ± 0.3 ^{ab*}	95.7 ± 0.5 ^{a*}	95.2 ± 1.0 ^{AB}	96.4 ± 0.2 ^B	93.4 ± 1.9 ^A
Total SFA	86.0 ± 0.5 ^a	91.4 ± 0.3 ^b	94.4 ± 0.8 ^b	86.0 ± 1.9 ^A	91.7 ± 0.5 ^B	92.5 ± 1.7 ^B
Total MUFA	95.6 ± 0.4 [*]	95.7 ± 0.5 [*]	95.3 ± 0.3 [*]	93.3 ± 0.7	95.1 ± 0.1	94.4 ± 1.0
Total PUFA	98.4 ± 0.3 [*]	98.1 ± 0.2 [*]	97.1 ± 0.2 [*]	96.9 ± 0.6	97.5 ± 0.1	96.4 ± 1.0
Total n-3	98.6 ± 0.3 [*]	98.5 ± 0.1 [*]	97.8 ± 0.3 [*]	97.1 ± 0.6	98.0 ± 0.1	96.9 ± 1.1
Total n-6	97.3 ± 0.2 [*]	97.2 ± 0.4 [*]	96.5 ± 0.2 [*]	95.9 ± 0.5	96.5 ± 0.1	95.9 ± 0.9
Total FA	93.2 ± 0.4 ^a	94.4 ± 0.3 ^{ab}	95.2 ± 0.5 ^b	92.1 ± 0.9 ^A	94.2 ± 0.2 ^B	93.9 ± 1.0 ^B
Total lipid	91.3 ± 0.4	92.7 ± 0.3	92.8 ± 0.8	90.3 ± 0.7	91.9 ± 0.3	91.5 ± 1.2

¹ Values (mean ± SE, n = 3) in the same row within the same water temperature with different superscripts are significantly different ($P < 0.05$). An asterisk (*) denotes significant differences between temperature treatments ($P < 0.05$).

² See footnote of Table 2 for description of abbreviations. FA = fatty acids.

Table 7

Summary of two-way ANOVA of the effect of diet, rearing temperature and their interaction on the apparent digestibility of fatty acids in rainbow trout.

Fatty acid	Source of variation ^a		
	Diet	Temperature	Diet x Temp. interaction
14:0	F = 5.8, P < 0.05	F = 5.5, P < 0.05	F = 0.1, P = 0.88
15:0	F = 14.3, P < 0.05	F = 7.7, P < 0.05	F = 5.5, P < 0.05
16:0	F = 28.4, P < 0.05	F = 0.8, P = 0.38	F = 0.8, P = 0.46
17:0	F = 30.8, P < 0.05	F = 0.9, P = 0.36	F = 0.3, P = 0.76
18:0	F = 23.1, P < 0.05	F = 1.4, P = 0.27	F = 0.5, P = 0.61
22:0	F = 2.6, P = 0.11	F = 0.0, P = 0.88	F = 0.5, P = 0.64
16:1n-7c	F = 3.9, P < 0.05	F = 6.4, P < 0.05	F = 0.1, P = 0.89
18:1n-9c	F = 0.4, P = 0.66	F = 7.4, P < 0.05	F = 1.4, P = 0.28
18:1n-7c	F = 0.7, P = 0.50	F = 3.3, P = 0.09	F = 2.0, P = 0.17
20:1n-9	F = 2.8, P = 0.10	F = 8.7, P < 0.05	F = 0.1, P = 0.90
20:1n-7c	F = 0.3, P = 0.77	F = 1.7, P = 0.21	F = 1.3, P = 0.32
22:1n-11c	F = 4.2, P < 0.05	F = 12.9, P < 0.05	F = 0.5, P = 0.62
24:1n-9c	F = 4.2, P < 0.05	F = 4.1, P = 0.07	F = 0.5, P = 0.62
18:2n-6	F = 0.7, P = 0.52	F = 7.6, P < 0.05	F = 0.9, P = 0.43
18:3n-3	F = 4.4, P < 0.05	F = 0.2, P = 0.64	F = 2.1, P = 0.16
20:5n-3	F = 1.3, P = 0.30	F = 5.3, P < 0.05	F = 1.2, P = 0.32
22:5n-3	F = 0.2, P = 0.83	F = 0.8, P = 0.38	F = 1.3, P = 0.30
22:6n-3	F = 5.8, P < 0.05	F = 11.8, P < 0.05	F = 0.2, P = 0.80
Total SFA ^b	F = 22.4, P < 0.05	F = 0.4, P = 0.56	F = 0.8, P = 0.49
Total MUFA ^b	F = 1.3, P = 0.30	F = 7.0, P < 0.05	F = 1.2, P = 0.34
Total PUFA ^b	F = 3.0, P = 0.09	F = 6.2, P < 0.05	F = 0.8, P = 0.50
Total n-3	F = 1.7, P = 0.22	F = 5.8, P < 0.05	F = 0.6, P = 0.58
Total n-6	F = 1.0, P = 0.40	F = 5.8, P < 0.05	F = 0.6, P = 0.54
Total fatty acid	F = 5.3, P < 0.05	F = 2.9, P = 0.11	F = 0.5, P = 0.63
Total lipid	F = 2.9, P = 0.10	F = 3.4, P = 0.09	F = 0.1, P = 0.94

^a Significance probability (P) associated with the F-statistic.

^b See footnote of Table 2 for description of abbreviations.

affected the AD of all fatty acids except 16:0, 17:0, 18:0, 22:0, 18:1n-7, 20:1n-7, 24:1n-9, 18:3n-3 and 22:5n-3. The AD of total SFA and total fatty acids were not significantly affected by water temperature. An interaction between diet and temperature effects was only found for 15:0.

4. Discussion

Higher digestibility of PUFA followed by MUFA and lastly of the SFA in rainbow trout observed in the present study was similar to trends reported in other studies (Olsen et al., 1998; Caballero et al., 2002; Ng et al., 2003b). Similarly, fatty acid digestibility in rainbow trout decreases with increasing chain length, but increases with increasing degree of unsaturation of the fatty acids as reported by Austreng et al. (1980), Johnsen et al. (2000) and Ng et al. (2003b). The absorption of total n-3 PUFA was consistently higher compared to total n-6 PUFA irrespective of dietary treatment, indicating the greater importance of n-3 long-chain PUFA to the nutritional requirements of rainbow trout. Differences in fatty acid absorption may also be due to the stereospecific position of the PUFA on the TAG structure (Christensen et al., 1995). Among other reasons, variations in the digestibility of individual fatty acids in fish have been attributed to different melting points of fatty acids (Austreng et al., 1980; Sigurgisladdottir et al., 1992), the tendency for long-chain SFA and MUFA to form insoluble soaps with divalent cations in the gut (Lied et al., 1987) and lipolytic enzyme specificity for PUFA (Koven et al., 1994).

High SFA content of CPO may reduce fatty acid digestibility and subsequent energy availability for farmed fish when incorporated at high levels in their diets. In a pilot scale study conducted with Atlantic salmon in Norway, Ng et al. (2004a) observed that increasing dietary CPO levels and decreasing water temperature significantly reduced the AD of SFA. The AD of MUFA and PUFA were only affected in salmon fed the 50% CPO diet but not at lower dietary levels tested (Ng and Gibon, 2010). In a feeding trial conducted with rainbow trout, it was

observed that increasing dietary CPO level (0 to 20% w/w) and decreasing water temperatures (15, 10 and 7 °C) significantly increased TAG content in trout fecal lipids, with SFA constituting more than 60% of excreted fatty acids (Ng et al., 2003b). The reduction in AD of SFA was therefore due in part to the increasing resistance of dietary TAG to digestion. Torstensen et al. (2000) reported that AD was significantly lower for all fatty acids except the n-3 fatty acids in Atlantic salmon fed a palm oil-based diet for 21 weeks at water temperatures of 8.0 ± 0.4 °C. Menoyo et al. (2003) reported that extruded salmon diets coated with only fish oil or fish oil partially replaced by palm stearin (8.9%) led to significant reduction in total lipid and SFA digestibility but not total MUFA and PUFA digestibility in large Atlantic salmon fed these diets in water temperatures ranging from 6.5 to 12.2 °C. These and other studies generally indicated that increasing dietary palm oil levels and decreasing water temperature significantly reduced the digestibility of SFA, but under normal circumstances has minimal impact on the AD of MUFA and PUFA.

In the present study, instead of CPO or other palm oil TAG fractions, rainbow trout was fed diets with added PFAD which consisted mainly of FFA. This is a novel approach in introducing a palm oil-based lipid source in salmonid diets which has higher levels of SFA compared to CPO but with most of the SFA occurring in free form. In this instance, we observed that increasing dietary PFAD did not reduce total lipid digestibility and actually improved total SFA digestibility in trout (Tables 6 and 7). The AD of MUFA and PUFA were generally not affected by dietary PFAD levels. When presented in free form, SFA seem to be easily absorbed by rainbow trout despite the increasing levels of dietary SFA concomitant with the increasing supplementation of dietary PFAD. The results from the present study are therefore in direct contradiction to the generally held view that the AD of lipids are negatively correlated to the dietary inclusion level of SFA (Caballero et al., 2002; Menoyo et al., 2003; Ng et al., 2004a). Based on a meta-analysis of data from several published studies with salmonids, Hua and Bureau (2009) predicted that when SFA exceeded 23% of the total fatty acids, AD of lipids decreases by 1.5% for every 1% increase in SFA content of the diet.

The results of the present study seems to suggest that other than the fatty acid composition of the lipid source, the dietary form of the lipid may also be an important factor influencing fatty acid digestibility. One possible explanation for the improvement in the AD of SFA observed in our study is that since FFA are more polar, they are able to form micelles more rapidly and with increased luminal micellar solubilization of these FFA, uptake by the gut enterocytes was enhanced. Variations in the digestibility of individual fatty acids in fish can also be attributed to the different melting points of fatty acids (Austreng et al., 1980; Sigurgisladdottir et al., 1992). In the present study, palmitic acid (16:0), the major SFA present in all diets, would have a lower melting point when presented as a FFA as compared to palmitic triacylglycerols (Bailey, 1950; Knoester et al., 1972) which should also improve the SFA digestibility of diets with higher FFA content.

The results of the present study further confirmed the important role of the trout pancreatic lipases in determining the digestibility of fatty acids. In a previous study, rainbow trout fed increasing dietary levels of CPO excreted increasing concentrations of undigested TAG in their feces (Ng et al., 2003b). In fish fed diets containing 20% CPO at water temperatures of 7 °C, orange colored oil-like substances were observed floating on the water surface of the tank. This was due to the increasing solidification of dietary oils in the digestive tract of trout when fed high levels of dietary TAG rich in SFA at decreasing water temperatures. Under these circumstances, a significant reduction in the AD of fatty acids, especially the SFA, was observed. In contrast, despite increasing dietary levels of SFA in the present study, fatty acid digestibility in rainbow trout either remained constant or improved. It would seem that when SFA are provided as FFA, high dietary levels can be incorporated into trout diets without any negative effects on lipid and fatty acid digestibility. When

present in free form, SFA do not need to be hydrolyzed by lipases and can be absorbed directly by fish. Free form SFA (as supplied by PFAD) was more easily absorbed compared to the same fatty acids present in the TAG molecule of dietary fish oil which probably accounted for the increased AD of SFA observed. Comparing the results of the previous study using CPO, which was presented in TAG form (Ng et al., 2003b), and the present study using PFAD which has a similar fatty acid composition (albeit slightly higher SFA) but in a FFA form, it would seem that differences in absorption of palm oil-based SFA could have been the result of impairment at the fatty acid hydrolysis step when high dietary levels of SFA are presented in rainbow trout diets in the form of TAG. This concurs with Olsen et al. (1998) when they concluded that both the rate of lipolysis and absorption may be rate limiting for long-chain SFA.

Glencross and Smith (1997) reported that the AD of fatty acids varied according to the form of the neutral lipid provided in the diet of the penaeid shrimp, *Penaeus monodon*. The AD of lipid was highest when the lipid source of similar fatty acid composition was presented as FFA compared to TAG or as ethyl or methyl esters. The AD of total SFA remained similar for both the FFA and TAG-based diets. Glencross and Smith (1997) suggested that the FFA lipid source may have mimicked the FFA products of lipase hydrolysis in shrimp enabling more rapid absorption of fatty acids as it bypasses the need for hydrolysis of TAG into FFA. FFA was suggested as a suitable source of supplementary fatty acids for penaeid shrimp. Sigurgisladdottir et al. (1992) reported no significant difference in absorption of fish oil fatty acids by Atlantic salmon when fed diets containing either the TAG or FFA form. This was not surprising considering the fact that only FFA were found in the fecal lipids indicating that dietary fish oil was totally hydrolyzed. In contrast, significantly higher levels of TAG were observed in the fecal lipids of trout fed the fish oil diet (0%PFAD) in the present study. Unlike the present study, improvement in SFA digestibility was not observed in palm-oil based diets fed to red hybrid tilapia (*Oreochromis* sp.) as compared to a fish-oil based diet (Bahurmiz and Ng, 2007). Despite the high FFA content of PFAD (92.2%) compared to CPO (4.8%) and refined palm olein (0.1%), the different FFA content did not significantly affect the nutrient digestibility of the palm oil-based diets. Differences in the effects of FFA on lipid and fatty acid digestibility may be species specific. Rainbow trout, being a carnivorous fish with a relatively short gut compared to tilapia, a planktivorous fish with an elongated gut, may derive more benefits in terms of lipid digestibility when dietary lipids are presented as FFA as compared to TAG. Nevertheless, it should also be pointed out that long chain free SFA tend to precipitate as calcium soaps in the gut which are then excreted, negatively affecting fatty acid availability (Lied et al., 1987). The positional distribution of fatty acids in dietary TAG also affects fatty acid hydrolysis and subsequent absorption in mammals (Mu and Hoy, 2004) and fish (Koven et al., 1994). Therefore, variation in digestibility estimates can be expected depending on the type of dietary TAG source used. The current very limited and seemingly contradictory data available on the use of FFA in aquafeeds points to the need for further research to better understand the role of different forms of dietary lipids as it relates to lipid utilization in the diets of farmed fish.

Most studies on the impact of water temperature on lipid digestibility have investigated the role of decreasing water temperatures (Windell et al., 1978; Olsen and Ringo, 1998; Ng et al., 2003b; 2004a) but the impact of elevated temperatures on lipid utilization is not well known. Decreasing water temperatures generally, though not always, tend to cause a reduction in AD of SFA which is further exacerbated by high dietary levels of SFA. Our study showed that increasing water temperatures did not cause an increase in lipid and fatty acid digestibility, with the possible exception of the fatty acid 14:0 and 15:0. On the contrary, the elevated water temperature of 20 °C tended to cause a reduction in the AD of most SFA, MUFA, PUFA and total lipid at each corresponding dietary treatment when

compared to trout maintained at 15 °C. Some of these differences in the AD of fatty acids were significantly different, albeit quantitatively small. It is possible that the observed lowered fatty acid digestibility at elevated water temperature are due to the trout lipases being adapted to function more optimally at lower water temperatures. Furthermore, increasing water temperature is also known to increase gastric evacuation and gut transit rate in rainbow trout (Fauconneau et al., 1983) and this would reduce the contact time between lipases and dietary lipids thereby negatively affecting hydrolysis of TAG. Like in the case of trout maintained at the optimal temperature of 15 °C, trout maintained at the elevated water temperature of 20 °C also showed improved total SFA digestibility when fed increasing dietary PFAD levels. Total lipid digestibility was also not negatively affected with increasing dietary PFAD levels. With global warming (Battisti and Naylor, 2009) and increasing water temperatures in many parts of the salmon farming world, the nutritional composition of salmonid feeds will need to be adjusted to ensure good fish growth and health. The present study represents an initial assessment of lipid utilization under elevated water temperature.

Data on the variation in fatty acid digestibility according to environmental temperatures encountered in the grow-out farms of salmonid fishes of various sizes is currently lacking and further research is needed to enable feed formulators to better calculate for dietary energy availability. Data on the effects of dietary lipid source and water temperature on the growth performance and body composition of rainbow trout obtained from the present study will be published elsewhere. Novel alternative lipid sources such as PFAD may hold the key not only in reducing feeding costs but also in adapting salmonid diets to the changing global climatic conditions.

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